Université de Toulouse

THÈSE

En vue de l'obtention du

DOCTORAT DE L'UNIVERSITÉ DE TOULOUSE

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Cotutelle internationale avec :

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Le vendredi 30 septembre 2016

Titre :

Spatiotemporal regulation of the Arbuscular Mycorrhiza Symbiosis establishment

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ED SEVAB : Interactions plantes-microorganismes

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Acknowledgments:

I first wanted to thanks the members of my PhD jury that have accepted to evaluate my work, and will take some of their summer time to read my work. I hope they will enjoy to read it especially if its on the beach or nearby a swimming pool.

Thank you again to the members of my PhD committee, Ms. Caroline Gutjahr and Mr. Benoit Lefebvre, for their time, their wise advise and the very interesting talks we had together.

Bien évidemment, tout cela n'aurait été possible sans mon cher directeur de thèse Mr. Guillaume Bécard, qui m'a fait confiance pour m'accepter en thèse. Et qui m'a permis notamment, grâce à ces sage conseil, et grâce bien sûr au soutient avisé de toute l'équipe myco, de réussir le concours de l'école doctorale, me permettant de quitter les froides contrées Flamandes pour le chaleureux sud de la France.

Guillaume, malgré les montagnes de travail qui te submergent constamment, et les nombreuses obligations auxquelles tu dois faire front, tu as toujours su trouver du temps et de l'écoute pour moi ainsi qu'à toutes les personnes qui viennent régulièrement te demander conseil. Tout cela avec calme, réflexion et bonne humeur. Ces nombreuses discutions, particulièrement autour du café-du-midi, m'ont beaucoup apprises, aussi bien sur le plan scientifique que culturel et on changées mes opinions sur de nombreux sujets (mais pas jusqu'à changer mon avis sur Nantes quand même).

Dans un autre style de management et de supervision tout aussi efficace et je dirais même complémentaire à celui de Guillaume, je voudrais remercier tout particulièrement Jean-Philippe Combier. Tu as été là au jour le jour durant ces trois années. A mon écoute, tu m'as fait confiance très rapidement, me soutenant et me permettant d'aller dans les directions qui me semblaient juste, tout en amenant toujours de nouveaux éléments à la discutions des résultats et à de nouvelles directions à prendre. Ta positivité constante et sans faille, ainsi que de ton dynamisme sont une bouée de sauvetage dans les moments de doutes et de résultats négatifs. Je sais que tu as été très déçu par mon manque d'assiduité au McDo mais je vais essayer de me rattraper tant bien que mal sur ma consommation de bonbon, personne n'est parfait. J'espère m'améliorer à l'avenir.

Bien sur la Science c'est avant tout un travail d'équipe et des discutions au coin d'un couloir qui peuvent changer le devenir d'une manip. Je voudrais donc remercier toute l'équipe myco qui a toujours été là pour discuter, confronter les hypothèses, tenter de résoudre les « pourquoi ça marche pas ».
Une pensé toute spéciale pour une personne alliant la lecture de Platon, avec une participation régulière au Hell-fest, un usage habile de la pipette et de moult techniques de biologie moléculaire, j'ai nommé le Dominique Lauressergues. Merci pour tous ces bons moments de rigolade, et d'aide plus sérieuse à la paillasse. J'aimerais pouvoir être aussi professionnel, compétent, enthousiaste et jeune dans ma tête quand j'aurais ton …expérience (pour ne pas dire âge).

Une pensé toute spéciale à quelqu'un qui nous a quitté trop vite (exil fiscal en suisse malheureusement), mais qui m'a permis de prendre pied au sein du laboratoire à mon arrivé. Merci pour toutes ces connaissances que tu m'as apporté, ces bons moments passés ensembles et ces soirées plus ou moins arrosées. Merci encore Mr. Jean-Malo Couzigou, j'espère pouvoir travailler de nouveau avec toi dans l'avenir.

En bien sur toutes les personnes de l'équipe myco, notamment Soizic pour sa rigueur scientifique et ses conseil d'une justesse sans pareil, mais aussi à Serge qui avec sa vision de drosophiliste m'ont beaucoup apporté, et bien sur tous les autres pour ces passionnantes conversation passée ensemble et tous ces bons moments.

J'ai aussi eu la chance d'évoluer dans un environnement riche et fort de nombreuses collaborations entre les différentes équipes du laboratoire. Je tenais à remercier le service imagerie de la FRAIB, qui nous permettent d'avoir accès à de nombreux appareilles performants. Sans compter, et c'est le plus important, leur grande implication, leur aide cruciale et leur enthousiasme pour nombre de nos projets. Merci particulièrement à Aurélie pour ces heures passée ensemble dans le noir des salles de microscopie.

Merci à ma famille qui m'a toujours soutenue, mais surtout qui s'est intéressé à mon travail. Cela qui m'a permis à de nombreuses reprises de faire un vrai exercice de vulgarisation de mon sujet. Ça a été et ça continuera d'être un réel .bonheur de répondre à vos questions.
Enfin merci à mon cher compagnon et à son soutien sans faille durant ces trois années de labeur. Pour son intérêt vis-à-vis de mon travail et son aide ponctuelle sur quelques manips du weekend.

Introduction

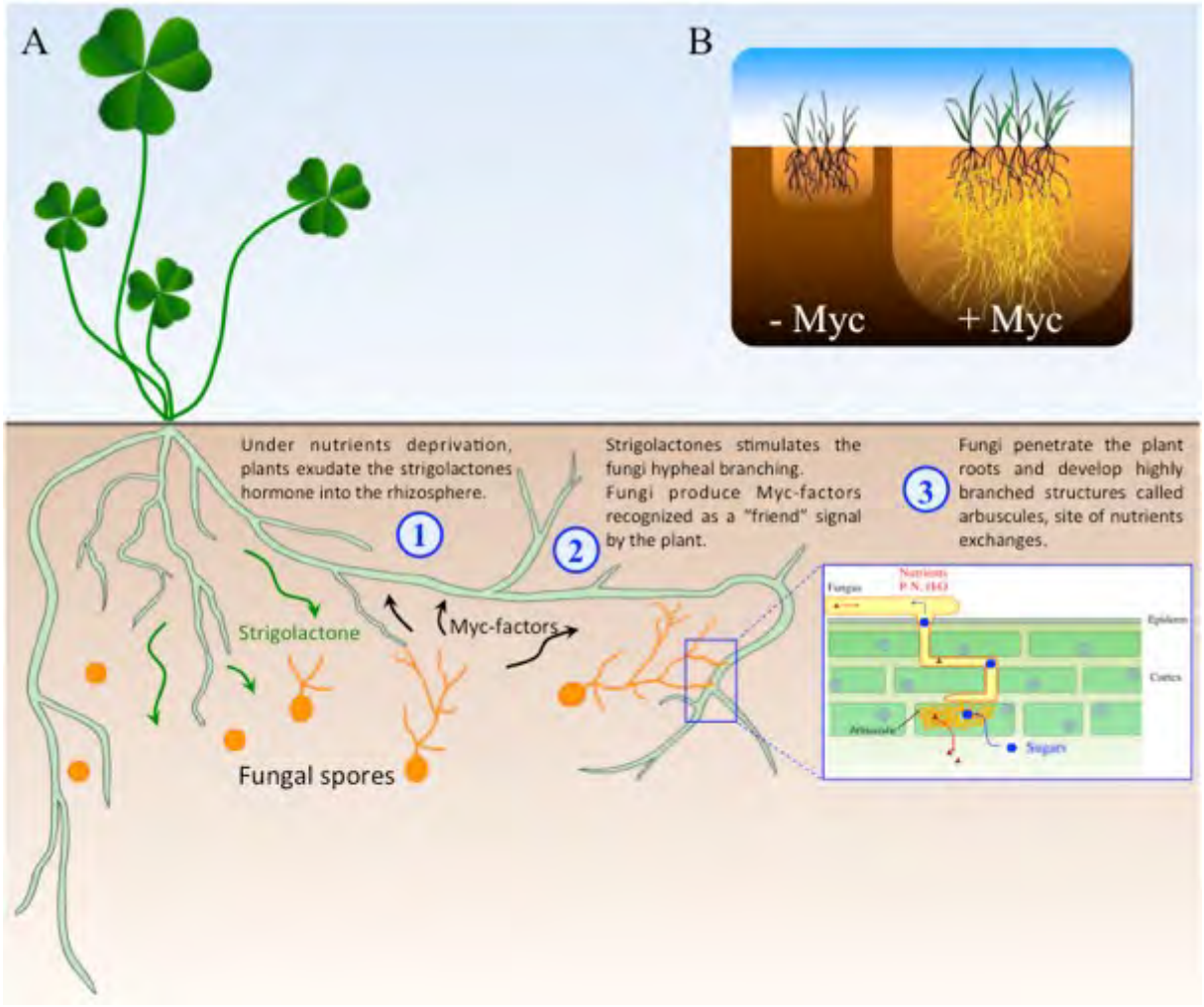


Figure 1: The mycorrhization process. (A) Schematic view of the primary dialogue between the plant and the fungus (1- 2) leading to the entrance and later the establishment of nutrient exchange structures called arbuscule (3). (B) Mycorrhized plants have, thanks to a dense hyphal web, access to a bigger soil volume, source of an improved mineral nutrition.

Throughout their lifespan plants encounter many challenges to overcome, from biotic stresses such as pathogen attacks, to abiotic stresses like nutrient or water deprivation. During their evolution plants have adopted several adaptive strategies to respond to these various stresses. One remarkable strategy is the establishment of a mutually beneficial interaction with arbuscular mycorrhizal (AM) fungi, a group of fungi that belong to a soil monophyletic fungal lineage the *Glomeromycota* (Harley & Smith, 1983; Schüβler *et al.*, 2001). This symbiosis has an extremely long plant-fungus history of co-evolution since its appearance 450 million years ago, and it has been proposed to have played a major role during plant colonization of land (Redecker *et al.*, 2000).

When the symbiosis is fully established, the fungus propagates within the root tissues and develops in root cells specific structures called arbuscules, where nutrients exchanges take place (Fig. 1 A). It also extents a dense hyphal web in the soil where it collects mineral nutrients, mostly phosphate (Harrison *et al.*, 2002; Paszkowski *et al.*, 2002; Nagy *et al.*, 2005; Bucher, 2007; Smith & Smith, 2011), but also ammonium, magnesium, sulfur, zinc, iron and water (Reviewed in Berruti *et al.*, 2016). Indeed, this extraradical mycelium, much more profuse and longer than root hairs, is able to penetrate smaller soil pores and to acquire nutrients from soil volumes that are otherwise inaccessible to roots (Smith *et al.*, 2000; Smith & Read, 2008; Allen, 2011) (Fig. 1 B). In exchange, the host plant feeds the fungus with sugars, unique sources of carbon for this obligate biotroph (Jakobsen, 1995; Smith & Read, 1997; Bonfante & Genre, 2010).

In addition to an improved nutritional supply, AM interaction provides other benefits to plants, such as a better drought and salinity tolerance (Augé, 2001, 2004; Porcel *et al.*, 2012) but also an increased resistance to some diseases (Pozo & Azcón-Aguilar, 2007).

Thus, AM symbiosis is of paramount importance for the proper functioning of plants, their productivity and therefore for terrestrial ecosystems. One can consider that, in natural environments, a non-mycorrhizal condition is the exception for the majority of plant species, especially because there is a marked diversity among AM fungal communities below ground, depending on plant diversity, soil type, season, or a combination of these factors (Smith & Smith, 2012).

1. Beneficial interactions start with an educated dialogue

The rhizosphere is a complex matrix made of several mineral components but above all, it contains a flourishing fauna and flora. Plant roots constantly encounter a plethora of bacteria and fungi and they have to distinguish between the beneficial one and the potential pathogens. Thus an intensive exchange of specific signals is necessary to ensure the recognition of each symbiotic partner and prepare them to a peaceful interaction.

1.1. Rhizospherical role of strigolactones

Under nutrient shortage, especially under phosphate deprivation, plant roots exude in the rhizosphere several classes of molecules and among them different types of strigolactones (SLs). These molecules are named from their first identified role as stimulants of seed germination of *Striga* parasitic weeds (Cook *et al.,* 1966) and from their lactone ring-containing chemical structure. Twenty years after the discovery of strigol, a germination stimulant of Striga seeds, researchers observed the capacity of root exudates of mycotrophic plants to stimulate the development of AM fungi, and especially hyphal branching (Graham, 1982; Elias & Safir, 1987; Bécard & Piché, 1989; Tawaraya *et al.,* 1996; Giovannetti *et al.,* 1999). After several years of investigation, two successive works from Akiyama et al. (2005) and Besserer et al. (2006) ended up with the characterization of SLs as the molecular signals produced both by mono- and dycotyledons and responsible for the induction of AM hyphal branching. Since then, extensive work has been done, using the synthetic SL analogue GR24, on the biological properties of these molecules.

It has been highlighted that upon GR24 treatment, AM fungi undergo an extreme stimulation of their mitochondrial metabolism such as, within minutes, an increase of NAD(P)H synthesis, NADH oxidase activity and ATP production (Besserer *et al.,* 2008). This stimulation of fungal oxidative metabolism was consistent with previous observations describing shape modifications and biogenesis of mitochondria upon treatments with root exudates or GR24 in *Gigaspora rosea* hyphae (Tamasloukht *et al.,* 2003; Besserer *et al.,* 2006). The proposed scenario is that when released in the rhizosphere SLs induce the germination of dormant fungal spores and the proliferation of germinating hyphae, which then will grow toward the emitting plant and eventually colonize the roots.

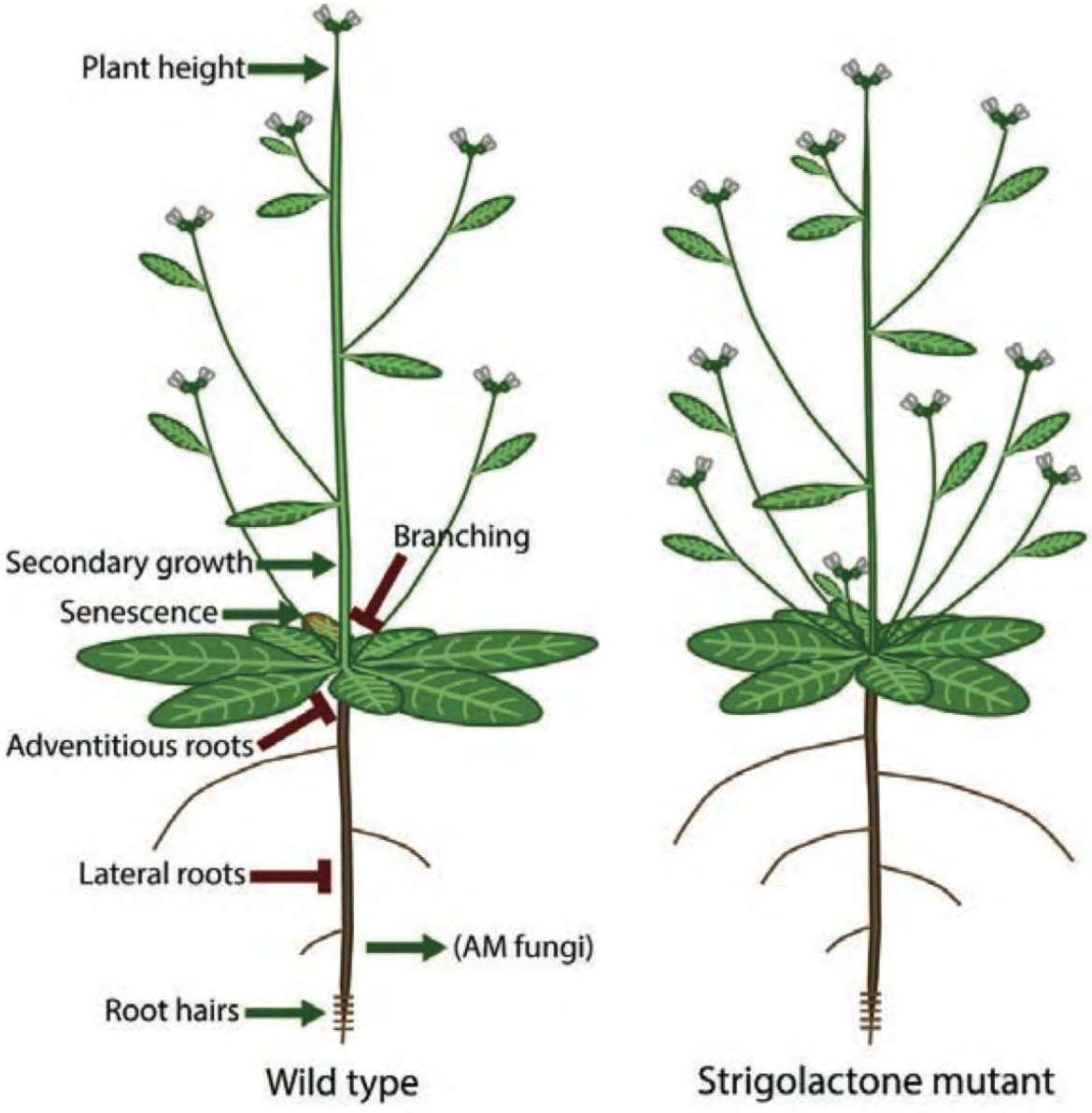


Figure 2: Proposed roles of strigolactones in adult plant growth and development (from Brewer *et al.*, 2012).

1.1.1 *Strigolactones as a plant hormone*

In addition to these rhizospherical roles, SLs have been later discovered as being a new class of plant hormones with a plethora of functions in plant development (Fig. 2). These new hormonal properties have been discovered by identifying several mutants impaired in SL biosynthesis or signaling. The restoration of wild-type shoot branching phenotype by GR24 application in overbranching pea, arabidopsis and rice mutant (*ccd8*) affected in SL synthesis demonstrated that SLs act as repressors of shoot branching by inhibition of lateral bud outgrowth (Umehara *et al.*, 2008; Gomez-Roldan *et al.*, 2008). Other actions of SLs on the aerial part of plants have been shown more recently, such as the stimulation of inter-fascicular cambium development (Agusti *et al.*, 2011). Furthermore, since the discovery that GR24 application reduces auxin transport (Crawford *et al.*, 2010) and triggers the rapid removal of the auxin efflux carrier PIN-FORMED 1 (PIN1) from the plasma membrane of the parenchyma cells of stem xylem (Shinohara *et al.*, 2013), one of the current hypothesis proposes that SLs act systemically to alter polar auxin transport in stems (Crawford *et al.*, 2010). In addition to this action, SLs might also have a role on a TCP transcriptional factor, PsBRC1/AtBRC1/OsTB1, repressing bud outgrowth through different pathway such as cytokinin and sucrose signaling (Minakuchi *et al.*, 2010; Braun *et al.*, 2012; Mason *et al.*, 2014; Rameau *et al.*, 2015).

Another important biological function of SLs is their roles in root development. Studies of several mutants impaired in SL synthesis or signaling, showed that under optimal growth conditions, SLs repress lateral root (LR) formation, promote root hair elongation and suppress adventitious rooting (Kapulnik *et al.*, 2011a,b; Rasmussen *et al.*, 2012; Liu *et al.*, 2013) (Fig. 2). A more recent study has highlighted the importance of SLs as repressor of LR initiation, by negatively influencing LR priming and emergence (Jiang *et al.*, 2016). Regarding their influence in lateral root development, SLs might act on auxin flux via PIN auxin-efflux perturbation (Koltai *et al.*, 2010a; Ruyter-Spira *et al.*, 2011). Since auxin is a key regulator of root development and its distribution determines lateral root position, initiation and elongation (De Smet, 2012), SLs might thus alter/regulate the auxin distribution pattern for lateral root formation.

This hormonal side of strigolactones hitherto has poorly been investigated in relation with the AM symbiosis. However during the later stages of the fungal colonization this hormonal side might play as well an important part that is still to be investigated.

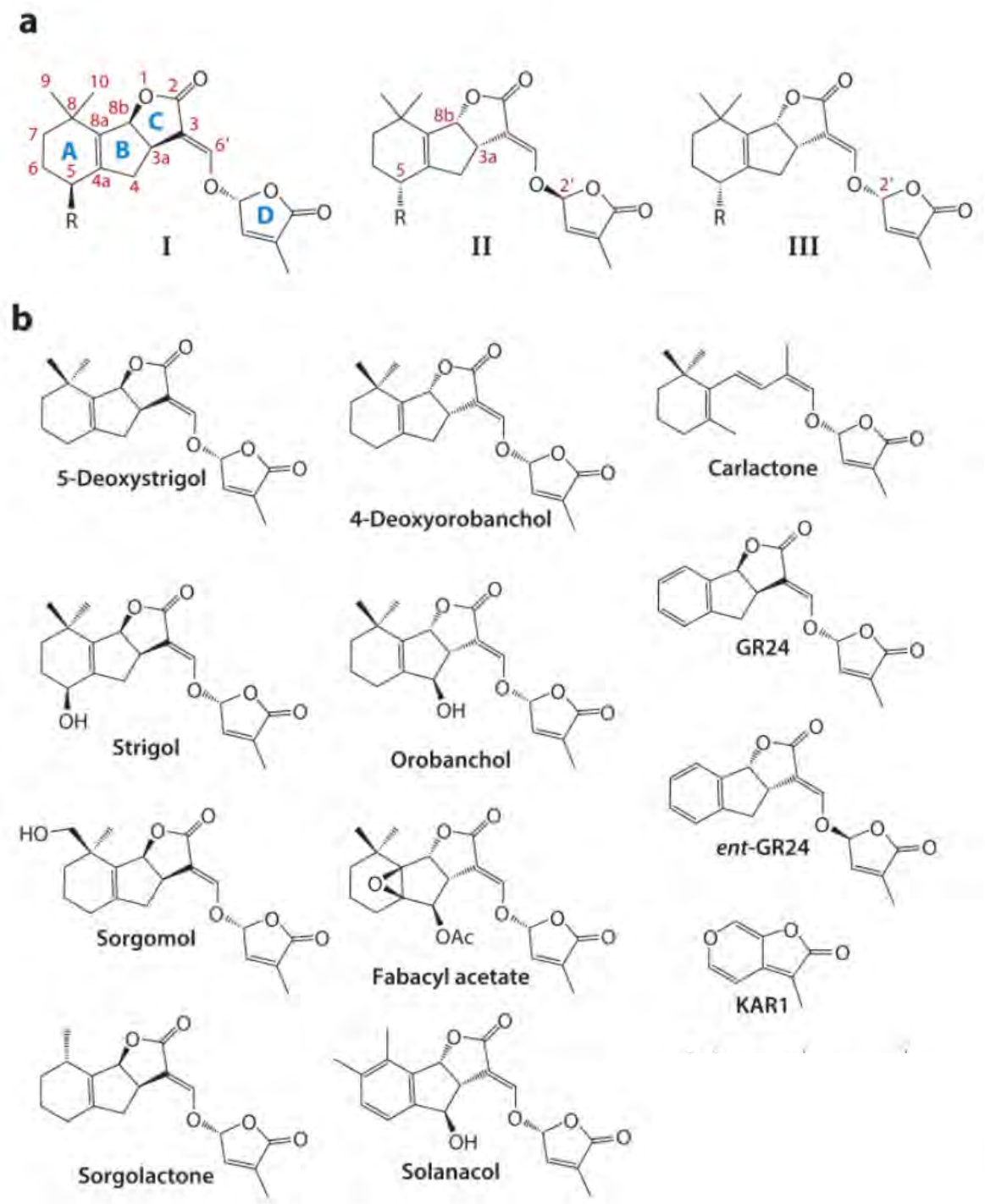


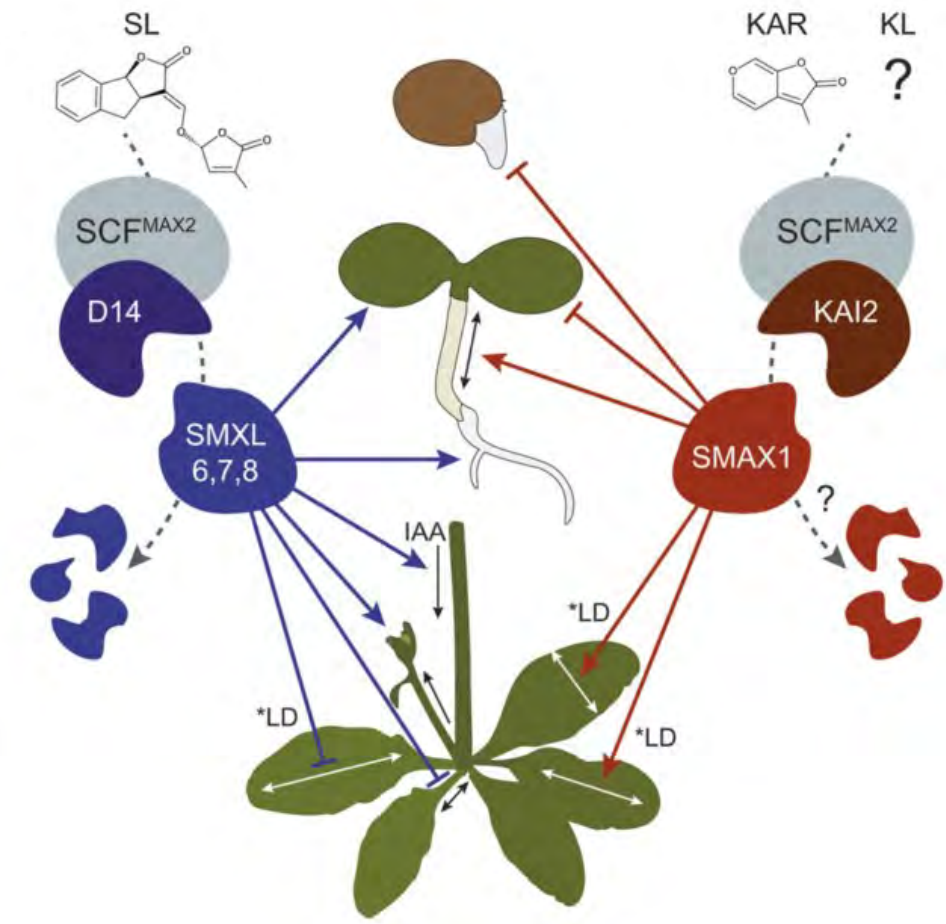
Figure 3: Structure of strigolactones and other compounds: (a) I schematic view of the four A-D rings, I and II represent the two enantiomers chemically produced II containing the S-configuration at the C-2' (not natural SL) and III the R-configuration at the C-2' (natural). (b) Different varieties of natural SLs. GR24 and ent-GR24, two enantiomers sold in the commercially available rac-GR24. KAR1 karrikin compound containing a D ring and able to interact with KAI2 the D14-like receptor (modified from Al-Babili *et al.*, 2015).

1.1.2 *Strigolactone structure and biosynthesis*

This class of carotenoid-derived compounds is characterized by a four ring structure (A-D), of which the C-D part is the most conserved and essential for biological activity, whereas the A-B rings show a wide diversity bearing various species-specific substitutions (Fig. 3 a, b) (Mangnus & Zwanenburg, 1992; Zwanenburg & Pospíšil, 2013). To date, at least 20 naturally occurring SLs have been identified and characterized in root exudates of various land plants (Xie & Yoneyama, 2010; Al-Babili & Bouwmeester, 2015; Tokunaga *et al.*, 2015). They can be separated in two types, strigol and orobanchol, according to the stereochemistry of the B‑C-ring junction, both types having a conserved R-configuration at the C-2'position (Fig. 3 a III). It has been clarified during the First International Strigolactone Congress (March 2015) that a special attention has to be paid to the use of racemic GR24 containing also S-configuration at the C-2' position (non present naturally) (Fig. 3 b left, GR24 and GR24-ent). Indeed it seems that these enantiomers could induce the karrikin-signaling pathway that share the same receptor interactor MAX2 (see the following part).

SL precursors are first synthesized in plastids from all-trans β -carotene via the action of an isomerase (D27) (Lin *et al.*, 2009; Alder *et al.*, 2012) and then two carotenoid cleavage dioxygenases (CCD7 and CCD8) (Alder *et al.*, 2012; Bruno *et al.*, 2014). The resulting carlactone is next transferred into the cytoplasm where it is subsequently processed by a cytochrome P450 family member (MAX1) to carlactonic acid and other yet unknown enzymes into orobanchol (Abe *et al.*, 2014; Zhang *et al.*, 2014a) (Diagram 1, green part, to unfold at the end of the introduction). Other unknown enzymes are also involved in the production of the large SL diversity found naturally (Fig. 3 b).

It is interesting to note that in Arabidopsis, the carlactonic acid is further transformed into the SL-like compound methyl carlactonoate (MeCLA) that interacts directly with the SL receptor *At*D14 (Abe *et al.*, 2014). Other SL-like compounds, with a carlactone-type structure lacking the canonical ABC-rings, have been discovered in different plants, highlighting the structural diversity of this class of compounds (Ueno *et al.*, 2011; Kim *et al.*, 2014). Once synthesized, all these compounds may be transported within the plant and in the rhizosphere. *Ph*PDR1, a member of the ABC family, has been identified as a potential SL transporter in petunia (Kretzschmar *et al.*, 2012; Sasse *et al.*, 2015).



SMXL 6, 7, 8

Promote:
- Branching
- Auxin transport
- PIN1 accumulation
- Lateral root density
- Cotyledon expansion

Repress:
- Petiole elongation
- Proximo-distal blade expansion in long days (*LD)

SMAXL 1

Promotes:
- Hypocotyl elongation
- Medio-lateral blade expansion (blade width)
- Elongation in long days (*LD)

Represses:
- Germination
- Seedling responses to light (reduces cotyledon expansion)

Figure 4: Strigolactones and Karrikin perception and signaling. Complex phenotypes observed in the *Athmax2* mutant are a combination of two different signaling cascades from SL and Karrikin. SMXL 6, 7, 8 are degraded via recognition of the MAX2/D14 complex while SMAX1 is degraded via the MAX2/KAI2 complex. Both signaling cascades result in distinct roles for the plant development listed below the scheme (Modified from Soundappan *et al.*, 2015).

1.1.3 *Perception and signaling*

The characterization of other mutants insensitive to SLs highlighted the importance of two major genes in the perception of SLs: the *D14* gene that encodes an α/β-fold hydrolase, which is able *in vitro* to hydrolyze GR24 into inactive ABC- and D-ring parts (Hamiaux *et al.*, 2012; Seto & Yamaguchi, 2014; Xiong *et al.*, 2014), and the *MAX2* gene that encodes a nuclear localized F-box protein. The presence of an F-box region on MAX2 suggests the implication of the SKP1-Cullin-F-box (SCF) complex, a ubiquitin E3-ligase complex which can catalyze polyubiquitination of substrates, thereby marking them for degradation by the 26S proteasome (Smalle & Vierstra, 2004). In 2013, the protein *Os*D53 was identified in rice and shown to be targeted for degradation after SL treatment in a MAX2 (*Os*D3) and D14 dependent manner. This first direct target of the D14/MAX2 complex belongs to the small family of proteins SUPPRESSOR OF MAX2 1 (SMAX1-like) and mutant of *d53* are SL insensitive and shows high tillering/branching (Jiang *et al.*, 2013; Zhou *et al.*, 2013) (Diagr. 1 purple)

The mechanism of SL reception by D14/MAX2 is still not fully understood, however it should be noted that the D14 SL receptor is closely related to the KArrikin Insensitive 2 (KAI2) receptor. *At*KAI2 perceives molecules with butenolide-containing rings, including the smoke-derived karrikin (KAR) compounds containing, as SLs, a lactone D-ring (Fig. 3 b, bottom right) (Waters *et al.*, 2012; Guo *et al.*, 2013; Smith & Li, 2014). The SL and KAR pathways modulate plant development in distinct ways but both require the F-box protein MAX2 to mediate their responses (Fig. 4) (Nelson et al., 2011).

Very interestingly, through an extensive analysis of loss-of-function mutants, it has been demonstrated that the Arabidopsis SMAX1-LIKE genes *SMXL6*, *SMXL7*, and *SMXL8* are co-orthologs of rice *D53* that promote shoot branching. SMXL7 is degraded rapidly after treatment with the synthetic strigolactone GR24 and like D53, SMXL7 degradation is MAX2- and D14-dependent. The loss of *SMXL6, 7, 8* in *max2* mutant, suppresses several other strigolactone-related phenotypes such as high branching, increased auxin transport and PIN1 accumulation. On the contrary SMAX1 does not seem to be related to the classical MAX2/D14 complex but rather to the MAX2/KAI2 complex and would therefore repress the karrikin signaling (Fig. 4) (Soundappan *et al.*, 2015; Wang *et al.*, 2015).

Finally SL signaling pathways appear to cross other hormonal signaling since a SL-dependent interaction between SLR1 a rice gibberellin signaling repressor (DELLA protein) and D14 was observed (Nakamura *et al.*, 2013). But also since BES1, a positive regulator in the brassinosteroid signaling pathway, was proved to be targeted for degradation via MAX2, in a SL independent manner (Wang *et al.*, 2013).

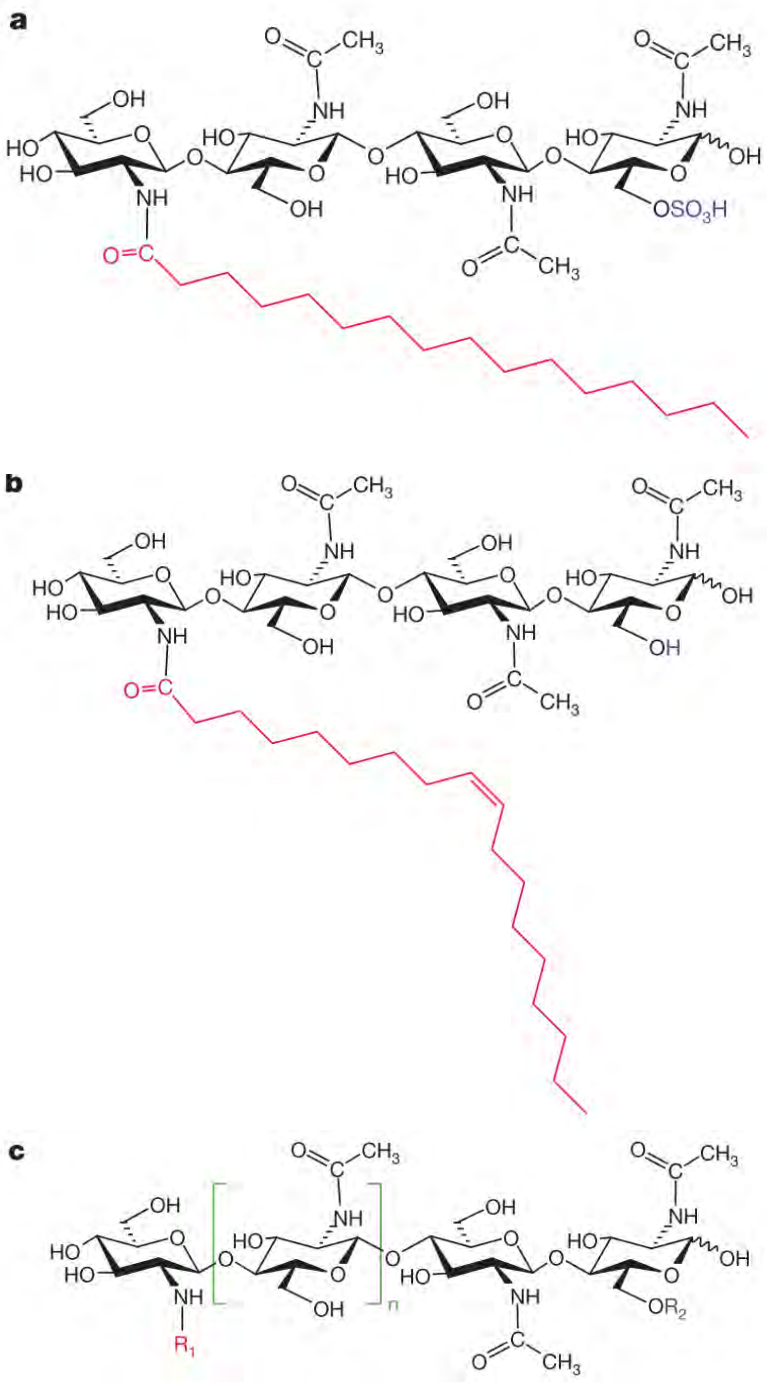


Figure 5: Structure of Myc-LCOs and COs. (a, b) chemical structures of two major Myc-LCOs produced by *R. irregularis*, (a) LCO-IV(C16:0, S) and (b) LCO-IV(C18:1D9Z). c, General Myc-LCO structure, for COs R_1 =H. (from Maillet *et al.*, 2011)

1.2. Rhizospherical roles of Myc-LCOs

2.1.4 *The good answer to Strigolactones: the Myc-LCOs*

On their side AM fungi produce specific type of molecules which are lipo-chitooligosaccharides (LCOs) called Myc-LCOs. These molecules consist of β-1-4-linked N-acetyl glucosamines (GlcNac) with an acyl chain at the non-reducing residue. *R. irregularis* produces a mix of sulfated and non-sulfated tetrameric and pentameric LCOs with either an oleic (C18:1) or palmitic (C16:0) acid (Fig. 5 a b) (Maillet *et al.*, 2011). Myc-LCOs have been shown to stimulate the mycorrhization process including in non legumes (Maillet *et al.*, 2011). They also activate root development growth and branching (Maillet *et al.*, 2011; Sun *et al.*, 2015b,a; Tanaka *et al.*, 2015). Because *R. irregularis* produces different types of Myc-LCOs, it will be interesting in the future to investigate the role of these different compounds separately, including as regulators of the plant immune system (Liang *et al.*, 2014), and the occurrence of the distinct Myc-LCOs types during different pre-symbiotic and early/late symbiotic phases.

In addition to Myc-LCOs, short-chain chitooligosaccharides (COs) consisting of four to five GlcNAc residues without an acyl chain could also play a role in AM symbiosis (Fig. 5 c) (Genre *et al.*, 2013). Tetrameric and pentameric chitooligosaccharides have been identified in exudates of germinated spores and their secretion was induced by the application of strigolactones.

Interestingly, there is a close structural homology between Myc-LCOs and Nod factors produced by rhizobial bacteria, and studies are on going to identify the Myc-LCO biosynthesis genes in the recently released *Rhizophagus irregularis* genome (Tisserant *et al.*, 2013). Several homologous genes have been found but functional studies will be necessary to confirm these *in silico* predictions.

2.1.5 *From recognition*

The recognition of the right beneficial partner by both the plant and the fungus, and the acceptance of each other are complex processes (Kiers *et al.*, 2003, 2011), that we might expect to be highly regulated by a large set of conserved genes. Among them, some are also implicated in the rhizobial symbiosis and encode for proteins involved in the so called Common Symbiosis Signaling Pathway (CSSP) (Oldroyd, 2013) (Fig. 6). To trigger this pathway, Myc-LCOs released by AM fungi are thought to be perceived by specific LysM domain-containing

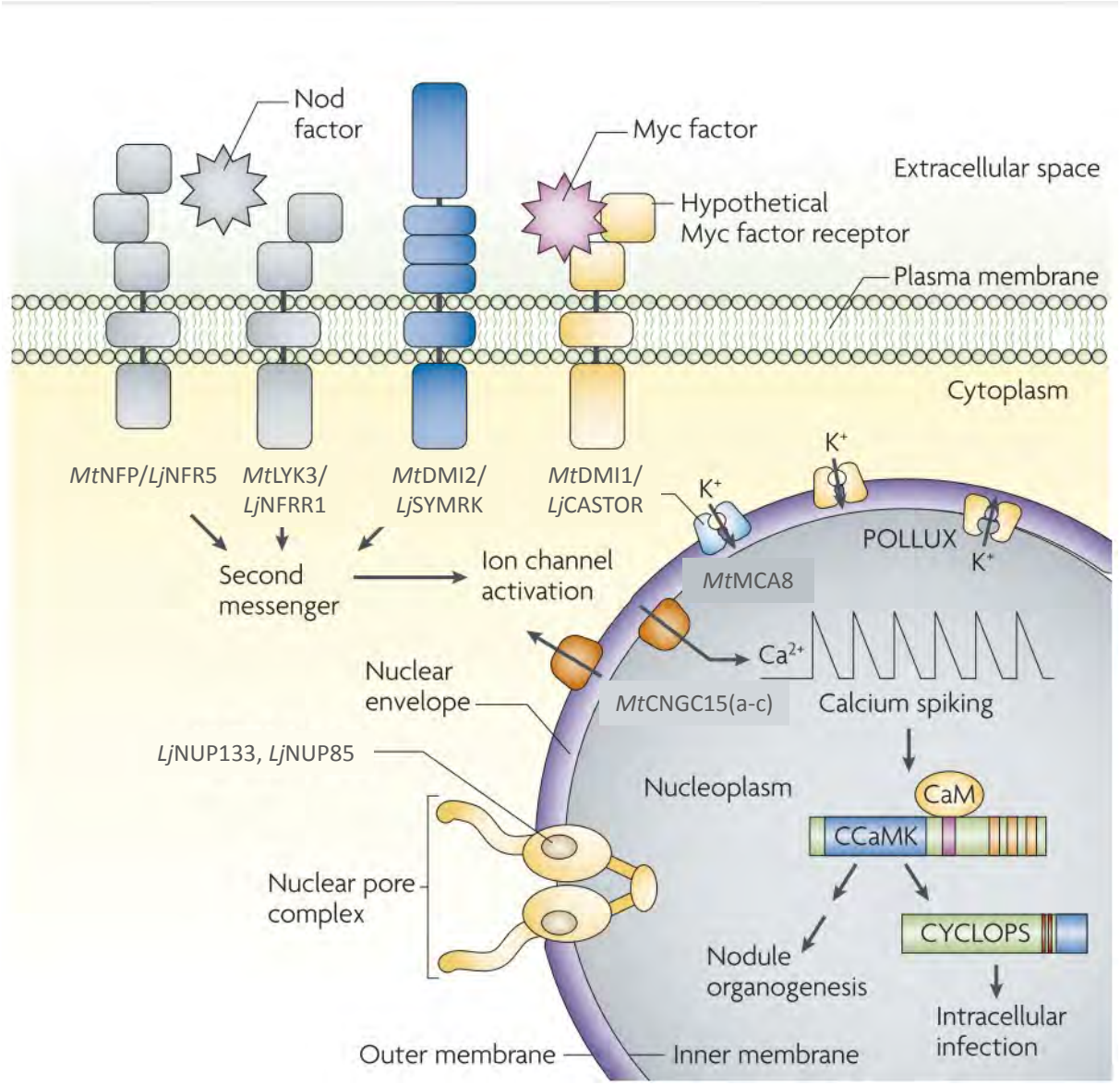


Figure 6: Common symbiosis signaling components for arbuscular mycorrhiza (AM) and root-nodule symbiosis. Perception of AM fungal or rhizobia-derived signals triggers early signal transduction, which is mediated by at least seven shared components. The symbiosis receptor kinase SYMRK acts upstream of the Nod factor- and Myc factor-induced calcium signatures that occur in and around the nucleus. Perinuclear calcium spiking involves the release of calcium from a storage compartment (probably the nuclear envelope and ER) through MtMCA8 and MtCNGC15 channels. The potassium-permeable channels CASTOR and POLLUX might compensate for the resulting charge imbalance. The nucleoporins NUP85 and NUP133 are required for calcium spiking, although their mode of involvement is currently unknown (Parniske 2008). The calcium–calmodulin-dependent protein kinase (CCaMK) forms a complex with CYCLOPS, a phosphorylation substrate, within the nucleus. Together with calmodulin, this complex might decode the symbiotic calcium signatures (Modified from Parniske 2008).

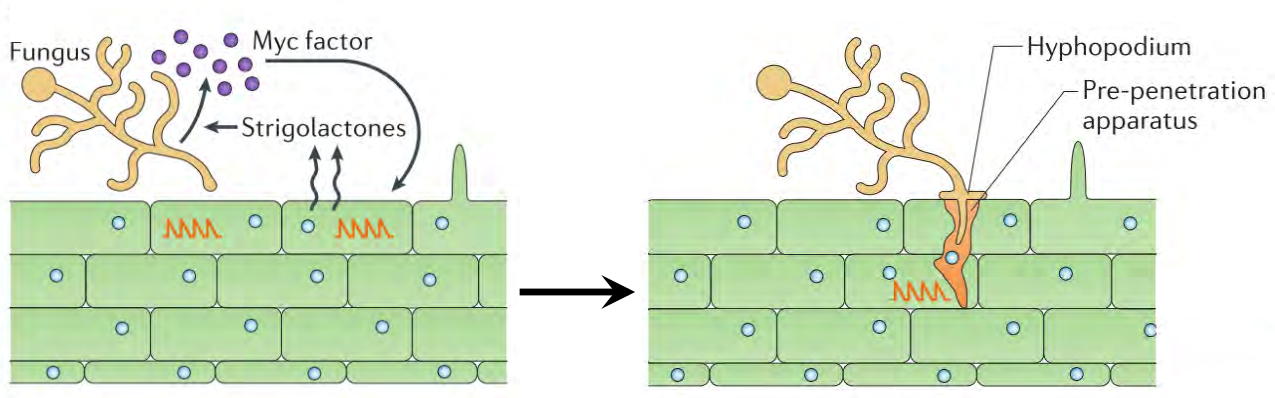
receptor-like kinases (LysM-RLKs) (not yet identified), like the Nod factors which are perceived by *Mt*NFP/*Lj*NFR5 (Nod Factor Perception) and *Mt*LYK3/*Lj*NFR1 (Genre *et al.*, 2013; Zhang *et al.*, 2015a). Interestingly Zhang *et al.* (2015) found in *Medicago truncatula*, *Lotus japonicum* and *Oryza sativa* that *MtLYK3*/*LjNFR1*/Os*CERK1* were required for AM colonization and perhaps encode proteins necessary for Myc-LCO perception. Once they have been perceived Myc-LCOs trigger the activation of calcium spiking in root cells (mainly epidermal), a characteristic feature of the CSSP (Maillet *et al.*, 2011; Sun *et al.*, 2015a). Then the CSSP is mediated by a plasma membrane LRR (leucine-rich-repeat) receptor kinase (*Mt*DMI2/*Lj*SYMRK) (Endre *et al.*, 2002; Stracke *et al.*, 2002) and a cation channel located at the nuclear envelope (*Mt*DMI1, *Lj*CASTOR and *Lj*POLLUX) (Ané *et al.*, 2004; Imaizumi-Anraku *et al.*, 2005; Peiter *et al.*, 2007; Riely *et al.*, 2007). Additionally calcium oscillation is generated by a two-component calcium transport, one responsible for calcium import into the nucleus (*Mt*MCA8) (Capoen *et al.*, 2011), and the other one for the calcium release comprising CNGC15 a, b and c (Charpentier *et al.*, 2016). These calcium oscillations are perceived by a nuclear-localized calcium-calmodulin-dependent kinase (*Lj*CCaMK/*Mt*DMI3) (Mitra *et al.*, 2004; Levy *et al.*, 2004) interacting directly with the transcription factor *Mt*IPD3/*Lj*CYCLOPS (Messinese *et al.*, 2007; Yano *et al.*, 2008; Chen *et al.*, 2008; Horváth *et al.*, 2011) (Fig. 6 and 7 B).

COs also trigger calcium oscillations through the CSSP in epidermal cells but independently of NFP or LYK3 (Chabaud *et al.*, 2011; Maillet *et al.*, 2011; Genre *et al.*, 2013; Zhang *et al.*, 2015a). Finally, in contrast to Myc-LCOs, short-chain chitooligosaccharides fail to stimulate formation of lateral roots in *M. truncatula*, but not in rice (Sun *et al.*, 2015b) (Fig. 7 B).

2.1.6 *To fungal entry*

After this exchange of plant and fungal signals leading to a mutual recognition, fungal hyphae in contact to roots differentiate into an attachment and penetration structure called hyphopodium (Fig. 7 A). This hyphopodium formation has been found to be negatively controlled by phosphate (Balzergue *et al.*, 2011). In addition, the successful entry seems to be dependent on Vapyrin gene made of a VAMP-associated protein and ankyrin-repeat domain (Pumplin *et al.*, 2010). In favorable condition, the plant produces in the epidermal cells in contact to hyphopodium a Pre-Penetration Apparatus (PPA). This PPA is made thanks to a specific cell cytoskeleton remodeling and allows the formation of an apoplastic tunnel in the

A



B

M. truncatula	*L. japonicus*	Protein type	Mycorrhizal phenotype	Calcium spiking		Nodulation	References
				Myc-LCOs	CO_4		
LYK3	NFR1	LysMdomain-containing receptor-like kinases	Less colonized	-	+	-	Zhang *et al.*, 2015
NFP	NFR5	LysMdomain-containing receptor-like kinases	Normal colonization	+	+	-	Zhang *et al.*, 2015
DMI2	SYMRK	LRR (leucine-rich repeat)-receptor kinase (membrane)	Impaired intracellular passage through the outer cell layer (or layers)	-	-	-	Genre *et al.*, 2013; Sun *et al.*, 2015
DMI1	POLLUX	Cation channel (nuclear)		-	-	-	Genre *et al.*, 2013; Sun *et al.*, 2015
	CASTOR	Cation channel (nuclear)		Unknown	Unknown	-	Imaizumi-Anraku *et al.*, 2005
MCA8	Unknown	Import calcium channel (nuclear)	Unknown	Unknown	Unknown	-	Capoen *et al.*, 2011
CNGC15(a-c)	Unknown	Export calcium channel (nuclear)	Reduced total mycorrhization	Unknown	Unknown	-	Charpentier *et al.*, 2016
DMI3	CCaMK	Calcium-calmodulin-dependent kinase (nucear)	Impaired in PPA formation, in intracellular passage through the outer cell layer and impaired arbuscule formation.	+	Unknown	+	Sun *et al.*, 2015
IPD3	CYCLOPS	Transcription factor	Impaired intracellular passage through the outer cell layer and impaired arbuscule formation.	Unknown	Unknown	+	Chen *et al.*, 2008

Figure 7: Schematic view of the pre-symbiotic and early stages of fungal colonization. (A) Perception of strigolactones by AM fungi promotes spore germination and hyphal branching. AM fungi produce mycorrhizal factors (Myc factors) which include lipochitooligosaccharides (LCOs) and oligosaccharides (COs), signals that activate the symbiosis signalling pathway in the root, leading to calcium oscillations (orange peaks). AM fungal invasion involves an infection peg from the hyphopodium that allows hyphal growth into the root epidermal cell. The route of hyphal invasion toward inner cell layers is predicted by a pre-penetration apparatus (PPA) in the plant cell (modified from Oldroy *et al.*, 2013). (B) The table summarizes the genes involved in the Common signaling pathway (CSSP), with their respective mycorrhizal phenotypes. + and – indicate if calcium spiking has been measured. "Nodulation" column indicates if the related mutants are able to perform rhizobial symbiosis (+ or -).

cell through which fungal hyphae grow and colonize the deeper root layers in the cortex. The PPA formation is highly dependent on the CSSP since the impairment of either one of the CSSP genes inhibits its formation (Fig. 7). Once they have reached the cortical cell layer, hyphae grow inter- and intra-cellularly along the roots and develop inside the cortical cells highly ramified structures, surrounded by the plasma membrane of the colonized cell, called arbuscules. These specific structures are the real headquarter of the bilateral trophic exchanges (very well reviewed in Casieri *et al.*, 2013) (Fig. 8).

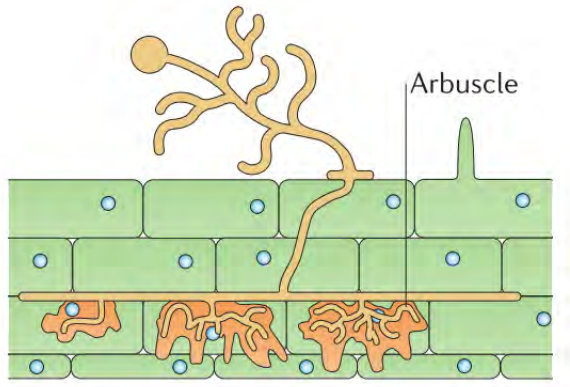
2. Arbuscular mycorrhizal symbiosis, a highly regulated partnership

It should not be forgotten that the establishment of such a symbiosis can represent a significant carbon cost for the plant (from 20 to 30% of its photosynthetic activity, (Peng *et al.*, 1993)). This is the reason why multiple mechanisms have been selected through evolution in order to allow the regulation of the fungus development inside the plant roots and temper its expansion. Indeed, it has been shown that AM fungi, less efficient in nutrient transfer, can be under-selected by the plant for more effective partners (Sanders, 2003; Kiers *et al.*, 2003, 2011; Javot *et al.*, 2007, 2011; Fellbaum *et al.*, 2012). Additionally, in fertile soil, where plants have easily access to nutrients, mycorrhization is reduced and even impaired if phosphate levels are high (Javot *et al.*, 2007; Breuillin *et al.*, 2010; Balzergue *et al.*, 2011). This is especially shown with plants mutated on the symbiotic phosphate transporter *MtPT4* and *OsPH1*. In these conditions phosphate from the phosphate cannot be transferred to the plant and as a result the development of fully branched arbuscules is impaired (Javot *et al.*, 2007; Yang *et al.*, 2012). Moreover, this control of root colonization seems to work in a symmetrical way. Indeed, when the sucrose transporter gene *GiMST2* of the fungus is silenced this prevents normal development of the fungus in the plant (Helber *et al.*, 2011). Helber et al. hypothesized that the induction of the phosphate transporter *MtPT4* is closely related to the induction of *GiMST2*. They suggested that a full development of the fungus in the root could only occur when a mutual benefit for both partners is fulfilled.

This control by the two partners of the compatibility of the interaction at several levels has therefore resulted from a double selection pressure and is what clearly distinguishes a symbiotic interaction from a pathogenic one. Presently, the mechanisms implicated in this balance are largely unknown.

Two recent phylogenomics studies have been conducted to identify highly conserved

A



B

M. truncatula	Protein type	Mycorrhizal phenotype	GUS pattern during myc	References
RAM1	GRAS transcription factor	Reduced hyphopodia, stunted arbuscules	Homogenous expression pattern in colonized root sections.	Gobbato *et al.*, 2012, Wang *et al.*, 2012, Hee-Jin *et al.*, 2015
RAM2	Glycerol3 phosphate acétyl transferase	Reduced hyphopodia, stunted arbuscules	arbuscule containing cells	Gobbato *et al.*, 2013
RAD1	GRAS transcription factor	Not fully branched arbuscules, less arbuscules and veshicles	Cells surrounding internat hyphea + arbuscule containing cells	Xue *et al.*, 2015
DIP1	GRAS transcription factor	Reduced general colonization	Unknow	Yu *et al.*, 2014
Vapyrin	Ankyrin domain protein	Reduced hyphopodia, stunted arbuscules	In epidermal and outer cortical cells beneath fungal hyphopodia and in the cortex during arbuscule formation, Expression coincident with hyphopodia is transient, and turns off after cortical colonization	Pumplin *et al.*, 2010
PT4	Phosphate transporter	Premature degradation of the periarbuscular membrane of arbuscules leading to symbiosis fail.	Expressed in fully functional arbuscules	Harrison et al., 2002 Helber et al., 2011
DELLA 1/2	GRAS transcription factor	Impaired in hyphopodium formation, but if fungal entry, normal arbuscules	Unknow	Floss et al., 2013

Figure 8: Schematic view of the established mycorrhization. (A) The fungus colonizes the plant root cortex through intercellular hyphal growth. Arbuscules are formed in inner root cortical cells from the intercellular hyphae (modified from Oldroy *et al.*, 2013). (B) The table represents a non-exhaustive list of crucial genes for arbuscule development and fully established symbiosis, including the localization of the related genes prom::GUS expression.

plant genes involved in AM symbiosis. They have led to the discovery of 174 (Delaux *et al.*, 2014) and 138 (Bravo *et al.*, 2016) genes strictly conserved in mycotrophic species and not related to nodulation (Delaux *et al.*, 2014; Bravo *et al.*, 2016). The valuable information provided by these two studies will be of great help in the future to investigate the subtle mechanisms of AM symbiosis establishment.

2.1. The concept of Autoregulation

In order to ensure a salubrious interaction, plants have to be aware of their mycorrhizal state. Therefore, there are several levels of local and systemic regulation in order to both promote and temper the colonization. The systemic regulations have been described by the use of split-root experiments consisting in the division of plant root systems grown in two separated compartments. The presence of AM colonized roots in one compartment leads, in the other compartment, to a strong decrease of root susceptibility for further colonization events (Vierheilig *et al.*, 2000; Vierheilig, 2004). This lower susceptibility of root colonization seems also to be highly dependent on the plant phosphate state since the supplementation of one compartment with phosphate leads to the suppression of colonization in the second compartment (Balzergue *et al.*, 2011).

This regulatory mechanism that limits the number of successful infection events is called autoregulation and plays a critical role during both mycorrhization (AOM) and nodulation (AON) (Staehelin *et al.*, 2011). It comprises a systemic, feedback inhibition initiated by early signals of the plant-microbe interaction suppressing subsequent infections. In studies of AON two key components of autoregulation have been described: CLAVATA1 (CLV1)-like kinase receptors called *Lj*KLV and *Lj*HAR1/*Gm*NARK/*Mt*SUNN (Searle, 2003; Mortier *et al.*, 2010; Miyazawa *et al.*, 2010; Lim *et al.*, 2011; Reid *et al.*, 2011; Okamoto *et al.*, 2013). Grafting and split-root experiments have revealed in soybean that NARK acts in the shoot, limiting infections systemically in the entire root system (Delves *et al.*, 1986; Caetano-Anollés & Gresshoff, 1990). Mutant plants with defective NARK are characterized by a supernodulating phenotype (Carroll *et al.*, 1985; Lin *et al.*, 2012) but also display an increased mycorrhizal colonization and higher arbuscule abundance (Pearson *et al.*, 1993; Vierheilig *et al.*, 2000; Zakaria Solaiman *et al.*, 2000; Shrihari *et al.*, 2000; Meixner *et al.*, 2005; Sakamoto & Nohara, 2009). Intriguingly, Nod factor application and cross-infections with rhizobia and AM fungi demonstrated initiation of a general autoregulation system by common signals cascade (Catford, 2003).

The *Lj*HAR1/*Gm*NARK/*Mt*SUNN kinases expressed in the shoot are, subsequently to symbiont entrance, activated by root-derived CLE peptides. However, even if some CLE peptides related to AON have been already identified (Reid *et al.*, 2011; MORTIER *et al.*, 2012), AM-induced CLEs are still unknown.

Acting downstream of *Lj*HAR1/*Gm*NARK/*Mt*SUNN in AON, a shoot-derived inhibitor (SDI) has been characterized biochemically as a heat-stable, ethanol-soluble, low-molecular weight molecule which is unlikely an RNA molecule or a protein (Kenjo *et al.*, 2010; Lin *et al.*, 2010). Little is known downstream of the SDI but TML (Too Much Love) an F-Box protein have been shown to be crucial for CLE-related pathway (Magori *et al.*, 2009; Takahara *et al.*, 2013). Finally, *Lj*NARK is described to affect phytohormonal balances including reduction of the shoot-to-root auxin transport and that of the jasmonic acid biosynthesis in the shoot, but it seems also to be involved in long distance transport of cytokinin (van Noorden, 2006; Seo *et al.*, 2007; Kinkema & Gresshoff, 2008; Sasaki *et al.*, 2014).

2.2. Strigolactone regulation

2.2.1 *Importance of the strigolactone signaling pathway for the mycorrhization*

Given the well-established role of exuded SLs in activating fungal growth before colonization it would be really interesting to investigate if the SL receptor complex, *Mt*D14/*Mt*MAX2 is required for AM development in planta (Yoshida *et al.*, 2012; Foo *et al.*, 2013b). Rice *dwarf3* (d3) as well as pea *ramosus4* (rms4) mutant roots (homologous genes of *MtMAX2*) present aberrant hyphopodia at the root surface with extremely rare penetrations into the inner cell layers. But the few arbuscules that might develop have wild type-like appearance suggesting that MAX2-mediated signaling is needed in the rhizodermis rather than in the cortex (Yoshida *et al.*, 2012). Surprisingly, mutants with a flawed D14 α/β hydrolase protein, the other component of the putative SL receptor complex, are even more strongly colonized than the wild-type (Yoshida *et al.*, 2012). This calls for an alternative receptor protein, which interacts with *Mt*MAX2/*Os*D3/*Ps*RMS4 during AM development and either binds SL or another small molecules. This opposite phenotype between *max2* and *d14* mutants might be explained by the possible interactions between MAX2 and KAI2 karrikin receptor and maybe other unknown proteins (Soundappan *et al.*, 2015).

Finally, recently in the rice mutant *hebiba*, the DWARF 14 LIKE gene has been found

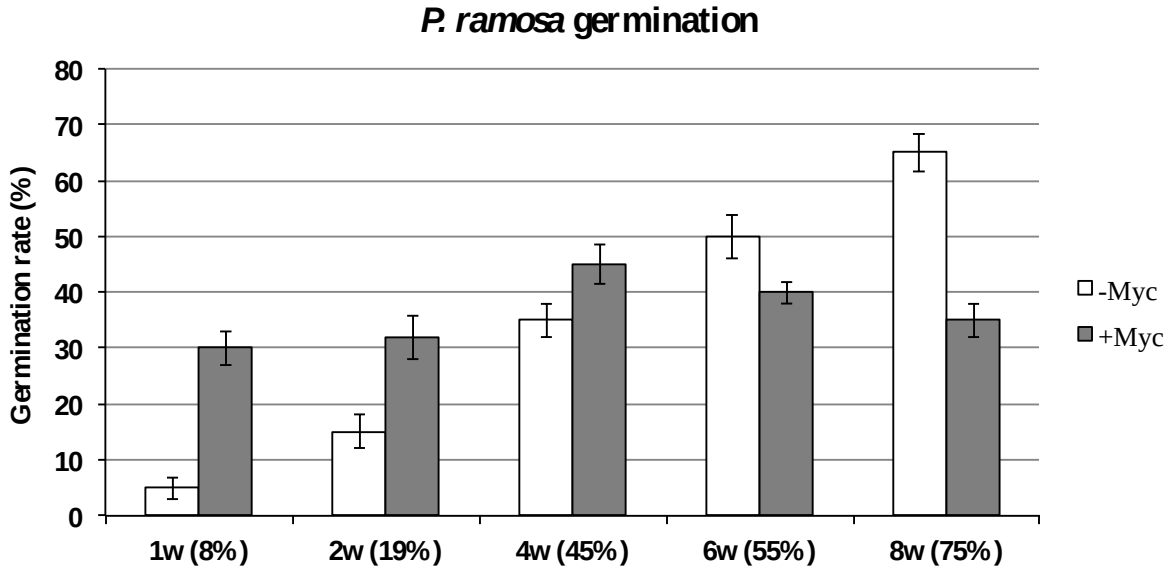


Figure 9: Summary of the results obtained in López-Ráez *et al.*, 2011 and 2014. Activity of tomato root extracts on seed germination of the parasitic weed *P. ramosa*, representing the SL content of the roots (measured by mass spectrometry in one of the two studies). On the bottom axis (w) stands for "weeks of tomato growth" and the (%) represents the total mycorrhization rate of the +Myc condition. In white, plants were not inoculated, while in grey plants were inoculated with *R. irregularis*.

to be crucial for fungal penetration and development in the roots (Gutjahr *et al.*, 2015). It encodes an α/β-fold hydrolase that is a component of the receptor complex involved in the detection of the smoke compound karrikin. This discovery adds a supplemental clue to the importance of the KAI2 signaling pathway in the regulation of the mycorrhization.

2.2.2 *Changes of strigolactone content through the colonization process*

During the slow mycorrhization process, from newly-colonized to well-colonized roots and fully established symbiosis, root SL content seems to be fluctuating.

Two successive studies from López-Ráez in 2011 and 2014 in Tomato have highlighted that during mycorrhization the root SL content increases transiently from 8 to 45% of total colonization but then decrease when roots are more colonized (from 55 to 75%) (Fig. 9). In non-colonized roots, SL content increases gradually over time mostly due to phosphate deficiency (Yoneyama *et al.*, 2007; López-Ráez *et al.*, 2008, 2011, 2014). It is still unknown if the decrease of SL content in mycorrhized roots is induced by the fungus itself or by phosphate delivered by the fungus. Nevertheless, these data provide evidence of a late regulation process that could have a great impact on the colonization balance.

2.2.3 *Strigolactone metabolic sides*

It has to be noticed that all-trans β -carotene, the initial substrate of SL biosynthesis is also a substrate of the ABsisic Acid (ABA) biosynthesis pathway and a precursor of mycorradicin (López-Ráez *et al.*, 2014; Walter *et al.*, 2015) (Diagr. 1 Red). However, even if some works have suggested the implication of D27 in the conversion of all trans-zeaxanthin or all trans-violaxanthin to 9-cis-zeaxanthin or 9-cis-violaxanthin, respectively, recent *in-vitro* studies of *Os*D27 activity did not provide any hint about the isomerisation of these two compounds (Al-Babili & Bouwmeester, 2015). On the opposite, new experiments have confirmed the possibility that CCD7 cleaves 9-cis-zeaxanthin leading to mycorradicin production but not the 9-cis-violaxanthin involved in ABA biosynthesis (Walter & Strack, 2011; Bruno *et al.*, 2014; Al-Babili & Bouwmeester, 2015). Nevertheless, disruption of ABA biosynthesis has been shown to affect negatively SL production (Matusova, 2005; López-Ráez *et al.*, 2010). For instance, root exudates of the ABA-deficient maize *vp14* and tomato *notabilis* mutants, have lower SL content. The decrease in SL content observed in *notabilis* and other ABA-deficient mutants might be caused by a lower transcript level of the SL biosynthetic genes *CCD7* and *CCD8*, suggesting that SL biosynthesis is regulated by ABA (Diagr. 1 red) (Matusova, 2005; López-Ráez *et al.*, 2010). Supporting this conclusion, the application of exogenous ABA led to a decrease in the transcript levels of *CCD7* and *CCD8* in Arabidopsis, which

was followed by a clear increase after 1 h of treatment (Ha *et al.*, 2014). Finally, tomato *sitiens* mutant reduced in ABA biosynthesis, and WT plants treated with ABA biosynthesis inhibitor, are less susceptible to mycorrhizal colonization (Diagr. 1 red) (Herrera-Medina *et al.*, 2007; Martín-Rodríguez *et al.*, 2011).

To our knowledge, mycorradicin roles in the AM symbiosis are still not known, although it has been shown that these molecules accumulate in cells containing aging arbuscules, and it has been proposed that this production may be responsible for the SL decrease, as a result of some metabolic rerouting (López-Ráez *et al.*, 2014).

2.2.4 *Implication of GRAS transcriptional factors NSP1 and NSP2*

The biosynthesis of strigolactones has been shown to be regulated by two transcriptional factors, NSP1 and NSP2 (NSP: Nodulation Signaling Pathway), which had been initially identified as essential for nodulation (Catoira *et al.*, 2000; Oldroyd, 2003; Kalo, 2005; Liu *et al.*, 2011). The *nsp1* mutant is not able to produce detectable SL amounts while *nsp2* is impaired in the conversion of orobanchol into didehydro-orobanchol recently identify as medicaol (Tokunaga *et al.*, 2015), which is the main strigolactone produced in *Medicago* ssp. (Liu *et al.*, 2011). Moreover, the decrease of SL production was correlated with a drastic down-regulation of two SL biosynthesis genes *D27* and *MAX1* in both mutants (Diagr. 1 green) (Liu *et al.*, 2011).

It has been shown that NSP1 is a DNA binding protein that binds to the promoter of some of the Nod factor inducible genes like *ENOD11*, *ERN1* and *NIN*. This protein seems to recognize the specific AATTT motif present in the promoter of these genes. NSP1 and NSP2 can form either homopolymers or heteropolymers, and the binding of NSP1 on different gene promoters requires the action of NSP2 which does not have DNA binding domains (Hirsch *et al.*, 2009). These transcriptional factors, present in single-copy in legumes, belong to the GRAS family and take part in the signaling cascades inducing both the rhizobial and the mycorrhizal symbiosis. In addition these proteins can form other heteropolymers with other GRAS TF (Smit, 2005; Herrera-Medina *et al.*, 2007; Hirsch *et al.*, 2009; Yu *et al.*, 2014; Park *et al.*, 2015). Finally, the transcription of *NSP1* is induced in mycorrhizal condition or by exogenous treatment with Myc-LCOs in an IPD3 dependent manner (Delaux *et al.*, 2013; Takeda *et al.*, 2013).

The lower SL production in *nsp1* is hypothesized to be responsible for the lower colonization phenotype observed in this mutant. More precisely it displays a lower frequency of infection perhaps due to a reduced SL-mediated stimulation of the fungus in the rhizosphere. In addition, the arbuscule abundance and the arbuscule shapes appeared normal meaning that

NSP1 may not be crucial for the later colonization stages and arbuscule morphogenesis (Delaux *et al.*, 2013). However, in the *nsp1* mutant of *Lotus japonicus*, arbuscule abundance was decreased and addition of the SL synthetic analogue GR24 was not able to rescue the *nsp1* phenotype suggesting other implications of this gene in the control of mycorrhization in this species (Takeda *et al.*, 2013).

It should be noted that Delaux *et al.* (2013) observed a lower colonization of *Mtnsp1* only when using a low inoculum of *R. irregularis* spores (400 sp/L). When working with 1200 sp/L, *Mtnsp1* displayed a WT mycorrhization phenotype. This shows the crucial importance to work with small inocula when studying very finely-tuned processes.

In 2011, a degradome analysis highlighted that *NSP2* was the target of a microRNA (miRNA), the miR171h (Devers *et al.*, 2011; Branscheid *et al.*, 2011). This miRNA is induced by Myc-LCOs mostly at the root tips and in the root elongation zone. Interestingly, the fungus rarely colonizes theses root apical parts. As expected, the overexpression of miR171h resulted in an inhibition of NSP2 and a lower mycorrhization. Additionally, the expression of a mutated version of *NSP2*, not regulated by the miR171h, increased the level of fungal colonization, which extended in 43% of the root tips instead of 4-7% in controls (Lauressergues *et al.*, 2012). Thus it appears that miR171h and NSP2 are playing a role in the spatial regulation of fungal colonization within roots.

It is highly tempting to speculate that NSP1, NSP2 and miR171h are linked together in the regulation of SLs via the control of *D27* and *MAX1*. SLs have so far been mainly studied for their action *ex-planta* in the rhizosphere but little is known about their late control of fungal growth inside the host roots (Yoshida et al., 2012).

2.3. Other regulations

3.2.5 *Interaction and importance of other GRAS in the control of mycorrhization*

As already said, *NSP1* and *NSP2* genes are part of a family of plant-specific GRAS transcription factors (TF), divided into 8 subfamilies that play important pleiotropic regulatory roles in root and shoot development: Gibberelic Acid (GA) biosynthesis, phytochrome A signaling pathways, abiotic stress, and of course symbioses (Di Laurenzio *et al.*, 1996; Peng *et al.*, 1997; Pysh *et al.*, 1999; Bolle *et al.*, 2000; Greb *et al.*, 2003; Tian *et al.*, 2004; Kalo, 2005; Smit, 2005; Fode *et al.*, 2008). Interestingly NSP2 interacts with the GRAS transcription factor Reduced Arbuscular Mycorrhization1 (RAM1) to induce RAM2. RAM2 is a glycerol-3-

phosphate acyl transferase (GPAT) responsible for de novo glycerolipid synthesis that participates in the biosynthesis of cutin and suberin (Beisson et al., 2007; Li et al., 2007). Mutation of RAM1 or RAM2 resulted in a strong defect of hyphopodia formation on the root surface during mycorrhizal colonization and to a defect of arbuscule development (Wang *et al.*, 2012; Gobbato *et al.*, 2012). Interestingly, translational GUS constructs showed that RAM1 is synthesized both at very early stages during hyphopodium formation (before fungal entry) and in fully colonized roots, while RAM2 is only present in the arbuscule-containing cells (Fig. 8 B) (Gobbato et al., 2013). This no-colocalition during the early steps of fungal entrance suggests that the phenotype of *ram1* cannot solely be explained by a non-induction of *RAM2* but also by other mechanisms. This is in agreement with new transcriptional studies presenting RAM1 as essential for the Myc-LCO-dependent pre-symbiotic reprogramming, and proposing that downstream of the CSSP, this GRAS transcription factor acts synergistically in the transduction of those diffusible signals that pre-announce the presence of the symbiotic fungus (Hohnjec et al., 2015).

3.2.6 *Implication of DELLA and GA*

Belonging to the GRAS family, DELLA proteins act as repressors of gibberellin (GA) signaling and thereby act as plant growth inhibitors (Hauvermale *et al.*, 2012). Four independent studies have now shown the importance of DELLA and GA for proper AM development (Foo *et al.*, 2013a; Floss *et al.*, 2013; Yu *et al.*, 2014; Takeda *et al.*, 2015). *M. truncatula* mutated in two of the three DELLA genes present in the genome (*della1*/*della2)*, displayed a strong a reduction of arbuscule number while the extent of root colonization was normal and intraradical hyphae seemed to proliferate even more than in the wild type (Floss *et al.*, 2013). The few arbuscules which were able to form in *della1*/*della2* developed to full maturity, indicating that DELLA proteins are required for the initiation of arbuscule formation but not for later stages of arbuscule development. However it is possible that a DELLA triple mutant of *Medicago* would display a more severe phenotype. Unfortunately, in both the *slender* rice1 (*slr1*) mutant, entirely DELLA-deficient, and the *la crys* DELLA pea double mutant, which presented similar AM phenotype, no precise information was provided on arbuscule morphogenesis (Foo *et al.*, 2013a; Yu *et al.*, 2014).

Interestingly, treatment of mycorrhized plants of *rice* with GA3 strongly inhibited infection point numbers, intraradical development of hyphae and arbuscule formation (Takeda *et al.*, 2015) (Diagr. 1 yellow right). Surprisingly, treatment with an inhibitor of GA biosynthesis only affected arbuscule formation. This is consistent with the fact genes involved

in GA biosynthesis and metabolism have been shown to be expressed in arbuscule containing cells (Takeda *et al.*, 2015). This indicates that a strict tuning of GA biosynthesis seems to be crucial for normal arbuscule formation. On the other hand, presence of GA does not seem to be essential for an efficient fungal entry or for hyphal development in the root, nevertheless it regulates negatively these two processes (Takeda *et al.*, 2015) (Diagr. 1 yellow right). In agreement with these studies a decrease of AM colonization (both in terms of frequency of colonization and arbuscule abundance) was observed in the tomato mutant (*procera*) with a GA-constitutive response (Martín-Rodríguez *et al.*, 2015).

More work is necessary to fully understand the implication of DELLAs and GRAS TF in the regulation of mycorrhization. A plethora of interactions and cross talks between these partners are not yet deciphered. For instance a new GRAS TF, DIP1, presenting a decreased AM phenotype when mutated, has been shown to interact both with DELLA and RAM1 (Yu *et al.*, 2014).

3.2.7 *Auxin signaling is crucial for arbuscule formation*

As already said, there is strong evidence that SL might influence auxin fluxes and distribution, leading to important hormonal cross talks. It is not surprising then that auxin also has an important influence on AM colonization. Recently the IAA reporter DR5-GUS was found to be specifically activated in arbuscule-containing cells of tomato, *M. truncatula* and rice indicating an elevated auxin-response associated with arbuscule development. Furthermore, in three independent studies, exogenous auxin treatment increased the mycorrhization rate (Hanlon & Coenen, 2011; Foo, 2013; Etemadi *et al.*, 2014) (Diagr. 1, orange middle).

In addition, overexpression of miR393, which targets the transcripts of the IAA receptor *TIR1/AFB*, caused an arrest in arbuscule formation (Etemadi *et al.*, 2014), suggesting that auxin receptor-mediated IAA perception is required for arbuscule development. During arbuscule development root cortical cells become strongly polarized, their cytoskeleton reorganizes and a distinct membrane domain, the peri-arbuscular membrane, forms and surrounds the arbuscule (Genre & Bonfante, 1998; Pumplin & Harrison, 2009). Since IAA

Hormone	Endogenous content in roots, myc vs non-myc	Effect of application on mycorrhization rate	Mycorrhization rate on different mutants	Key references
Ethylene	+	-	Mutants with more Ethylene production or increase ethylene responses => less mycorrhization	(Geil *et al.*, 2001; Geil & Guinel, 2002; Torres de Los Santos *et al.*, 2011; Fracetto *et al.*, 2013; Foo *et al.*, 2014)
Brassinosteriod	nd	nd	Mutant defective in BR synthesis => less mycorrhization	(Bitterlich *et al.*, 2014; Foo *et al.*, 2016)
Cytokinin	+	+	Cytokinin receptor mutant cre1 => no effect on mycorrhization	(Allen *et al.*, 1980; van Rhijn *et al.*, 1997; Ginzberg *et al.*, 1998; Laffont *et al.*, 2015)
Jasmonic acid	+	+ (Low [C]) - (High [C])	Mutants producing less JA or RNAi against AOC (JA biosynthesis gene) => less mycorrhization	(Regvar *et al.*, 1996; Hause *et al.*, 2002; Ludwig-Müller *et al.*, 2002; Vierheilig & Piché, 2002; Isayenkov *et al.*, 2005; Stumpe *et al.*, 2005; Meixner *et al.*, 2005; Tejeda-Sartorius *et al.*, 2008)
Salicylic acid	-	-	Mutant less producing SA => more mycorrhization at early stages Mutant over-producing SA => less mycorrhization at early stages After several weeks same colonization levels as WT	(Blilou *et al.*, 1999, 2000a,b; Ludwig-Müller *et al.*, 2002; Herrera Medina *et al.*, 2003)

Table 1: Implication of other phytohormones during the mycorrhization.

application can stimulate cytoskeletal rearrangement and local IAA maxima can lead to a TIR1-dependent re-polarization of cells (Nick *et al.*, 2009; Vineyard *et al.*, 2013) it has been proposed that TIR1/AFB-dependent IAA signaling mediates cytoskeleton re-arrangement and polarization of cortex cells during arbuscule formation (Gutjahr, 2014).

3.2.8 *On the importance of other phytohormones, only the surface has been scratched*

In addition to ABA, GA and Auxin other phytohormones have been studied in the context of mycorrhization: cytokinins, brassinosteroids, ethylene and the two defense related hormones salicylic acid and jasmonic acid. As described in table 1 these five hormones differently influence the mycorrhizal process. Interestingly, none of the mutants tested hitherto presented defect in fungal structures. They rather displayed differences in the colonization rate or in the abundance of arbuscules. This leads us to think that these hormones are playing some role, yet to be fully understood, in the general regulation of AM fungal development and that they may very well be involved in the autoregulation process.

Moreover it should be noticed that their content in the roots seems to change when mycorrhized (Table 1). These fluctuations can be due to a modification by the plant of its own hormonal metabolism in response to a new mineral state, or in response to the fungal presence. But these fluctuations could also result from the fact that the fungus highjacks the plant by interfering with its phytohormone signaling network, promoting its own growth, as some pathogens do (Reviewed in Kazan & Lyons, 2014). In the case of ethylene, cytokinin and jasmonic acid, whose content increases in mycorrhized roots compared to non-mycorrhized ones, it is tempting to speculate that the fungus itself may be able to synthesize them, or some analogous molecules, so that it will tune locally the hormonal balance of the plant in a suitable way for its development.

3. Doctoral work main goals:

As described above, there are many subtle molecular mechanisms and cross regulations between them that are necessary to maintain a perfectly balanced beneficial AM interaction. Most of these mechanisms are still unknown.

Because of the great difficulty to study spatiotemporally regulated processes, little is known about the role of genes that are non-crucial for the proper fungus morphogenesis but that are rather involved in the subtle fungal development/propagation inside the host roots.

This is the reason why in this doctoral work, we will investigate more thoroughly the role of the NSP1 and NSP2 GRAS transcriptional factor that have been only recently involved in the regulation of the colonization process (Maillet *et al.*, 2011; Delaux *et al.*, 2013).

- How these two genes are regulated before and during the AM symbiosis?
- What is their involvement in the regulation of SLs?
- We also wanted to precise the role and the spatiotemporal regulation of the miR171h that target *NSP2.*
- And finally, because in the team we could unravel the crucial role of the auxin signaling during the mycorrhization. We wanted to go further in this way by studying the role of an auxin signaling component that seems to also play a role this symbiosis.

Chapter 1:

The implication of NSP1 during the AM symbiosis

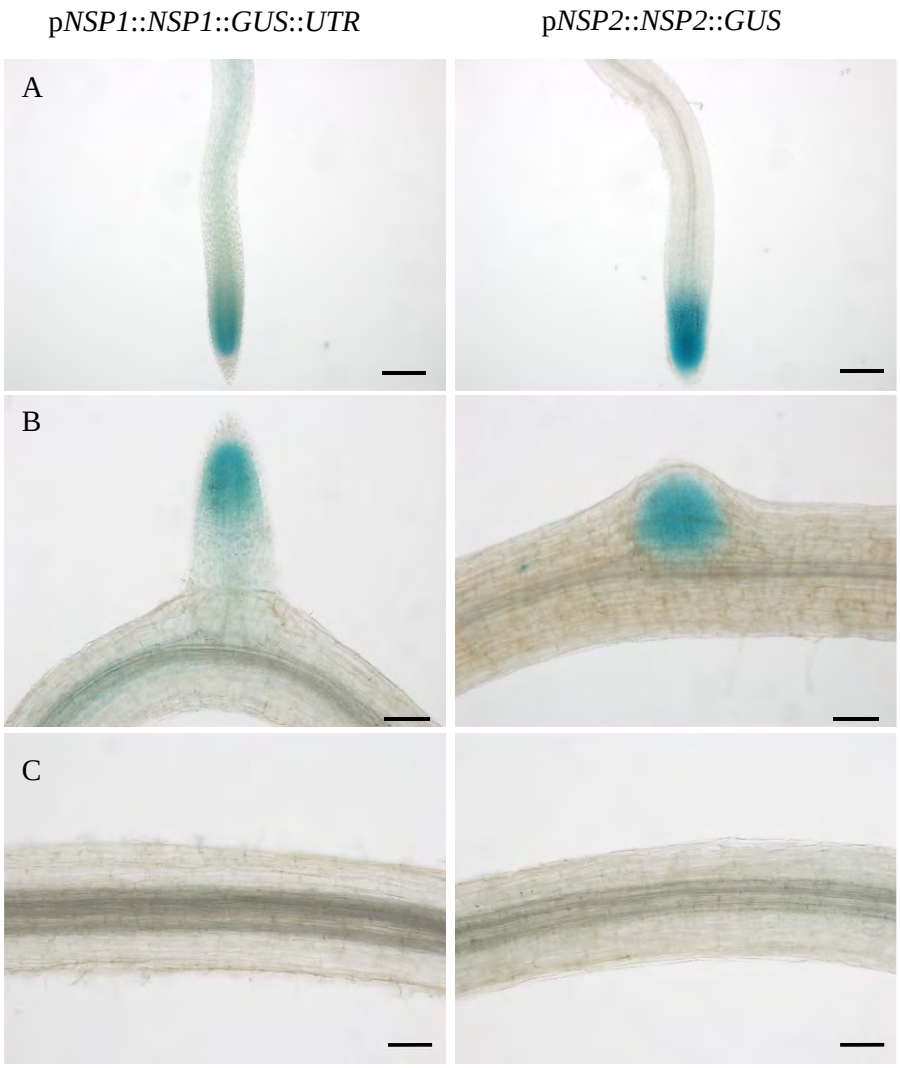


Figure 1: Expression patterns of NSP1 and NSP2 translational fusions in *Medicago* A17 WT hairy roots. (A) Both constructs are expressed in the developed root tip and (B) emerging lateral roots. No expression was visible in root portions far from the root tip (C). Scales A=200µm, B, C=100µm.

1. Results

1.1. NSP1 directly activates the expression of *D27* and *MAX1*.

It has previously been shown that expression of *D27* and *MAX1*, two genes involved in strigolactone biosynthesis, is controlled by the transcription factors NSP1 and NSP2 (Liu *et al.*, 2011). However, the biological relevance of this regulation, as well as its fine-tuning remain largely unknown. To have a better understanding of this regulation network, we first checked that the presence of NSP1 and NPSP2, co-localize with the expression of *D27* and *MAX1*.

We prepared constructs of *NSP1*- and *NSP2-GUS* translational fusions and verified that these constructs could complement respectively the *nsp1-1* and *nsp2-2 Medicago truncatula* mutants for both nodulation and mycorrhization. The translational fusion of *NSP1* and *NSP2* genes with the *GUS* reporter gene contained 3kb of their promoter at the 5' end and 3kb of 3' UTR. After *Agrobacterium rhizogenes* transformation of *M. truncatula nsp1-1* mutant with the *NSP1-GUS* translational construct, the chimeric plants were rescued for both nodulation and mycorrhization (Fig. S1). The *NSP2-GUS* translational construct could not, suggesting that other regulating DNA sequences were missing. We then tested other versions such as including the *GUS* sequence at the N-terminal part of *NSP2* or removing the 3kb containing the 3'UTR region and none were able to complement the *nsp2* mutant. Interestingly, analysis of the GUS expression pattern showed no expression of NSP2 in any conditions when the 3'UTR region was included, strongly suggesting that *NSP2* translation is highly regulated by unknown factor(s) and regulatory sequences. For the following analyses of NSP2 expression, despite the lack of complementation of the *nsp2* phenotype, we used the *NSP2* translational construct with the GUS sequence at the C terminal domain and lacking the 5'UTR region.

Analysis of the expression pattern in *M. truncatula* chimeric roots shows that both NSP1 and NSP2 are synthesized in root meristematic parts as well as in lateral root primordia (Fig. 1A, B). No translational expression of these two genes was found in roots far from root tips (Fig. 1C). This specific translational expression of NSP1 and NSP2 in the meristematic root zone is consistent with the transcriptional expression pattern of these two genes as described by Untergasser *et al.*, 2012.

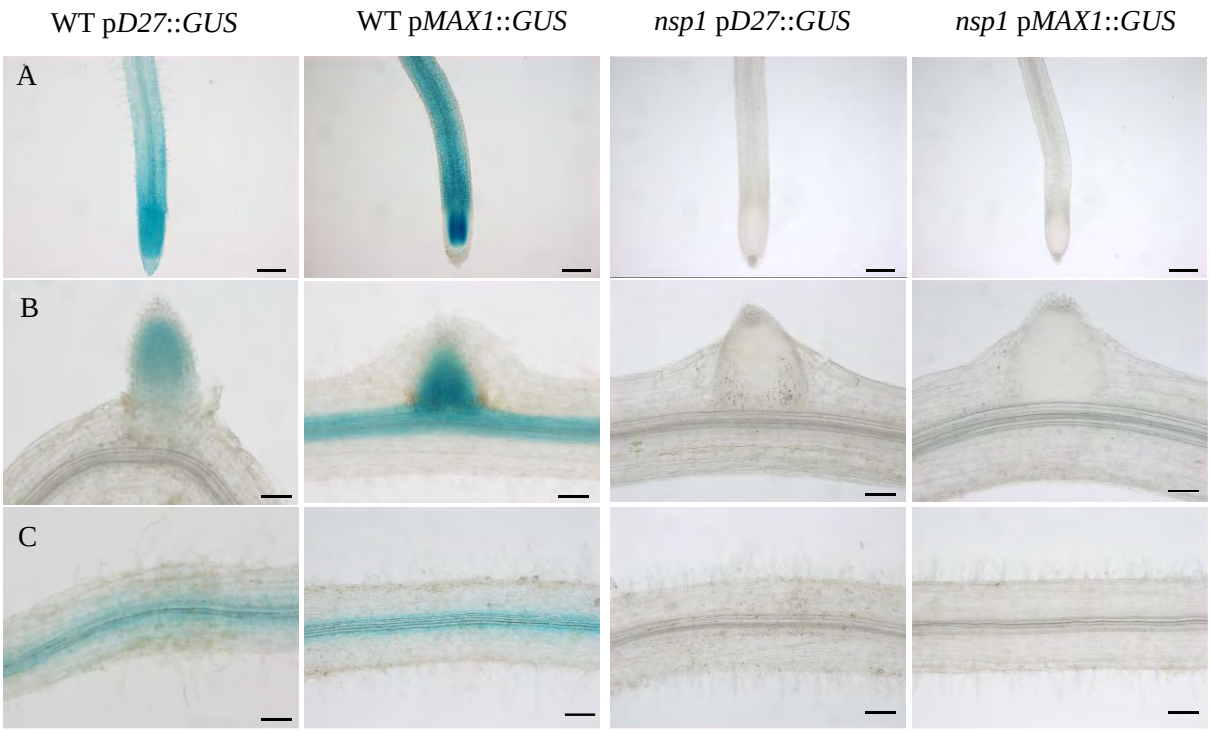
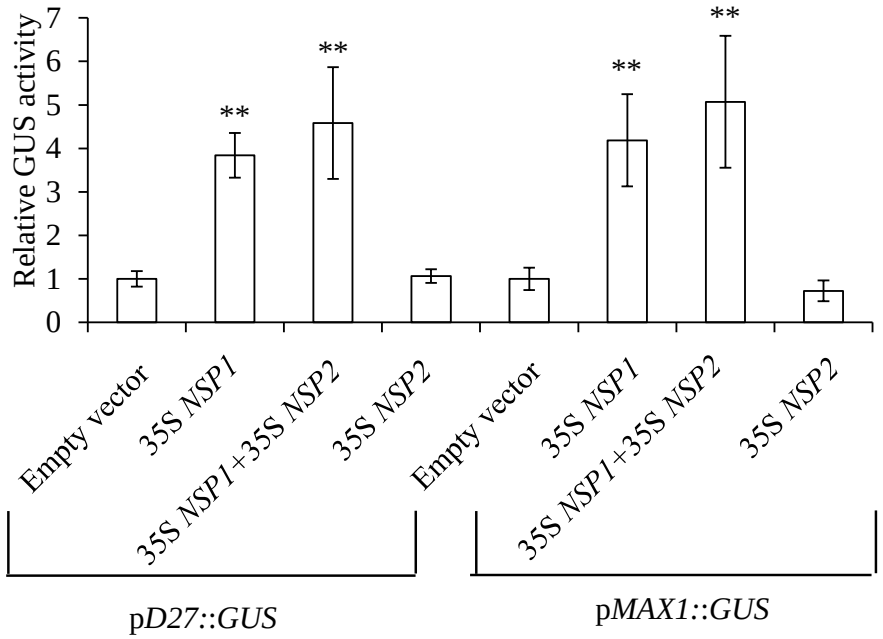


Figure 2: Expression pattern of *D27, MAX1* promoters in *Medicago* WT and *nsp1-1* hairy roots. GUS expression is only visible in the WT roots while in *nsp1* roots both promoters appear to be not expressed. (A) Expression in the root tip and the elongation zone. (B) Emerging lateral root and (C) root portion far from the root tip. Scales A=200µm, B, C=100µm.

We then analyzed the localization of *D27* and *MAX1* transcriptional expression. During the first steps of this work we used a construct containing 3kb of the promoter region of *D27* and *MAX1* fused to the GUS sequence. This construct was made to ensure the presence of every potential regulatory sequence. However, when expressed in *M. truncatula* chimeric roots both constructs revealed an extremely strong expression throughout the roots (less than 5mins in the GUS buffer to see well the blue staining). This too high expression did not allow us to analyze finely the spatiotemporal regulation of these genes. Hence, we used *D27* and *MAX1* promoter-GUS constructs with a 1 kb promoter that possess 9 putative NSP1 (AATTT) regulatory sequences for *D27* as described for in Liu *et al.*, 2011, and 5 in the *MAX1* promoter according to our analysis. Using these constructs we could observe a strong expression pattern of both *D27* and *MAX1* promoters in root apical zones and in lateral root primordia corresponding to the NSP1 and NSP2 expression (Fig. 2 A B). However, contrary to NSP1/NSP2, they are also expressed in vascular and cortical tissue (Fig. 2C), as it had already been shown for *D27* by Van zeijl *et al.* (2015).

Then, we expressed the *D27* and *MAX1* promoter-GUS fusions in the *nsp1-1* background mutant and observed no GUS staining, suggesting that NSP1 was responsible for *D27*/*MAX1* induction (Fig 2A, B and C). However it is intriguing that the expression of *D27* and *MAX1* was also lost in the central cylinder and the cortical cells where NSP1/NSP2 did not seem to be expressed (Fig. 1). It is possible that the expression of these transcription factors is rather low in these tissues and could have been detected by longer GUS staining.

A



B

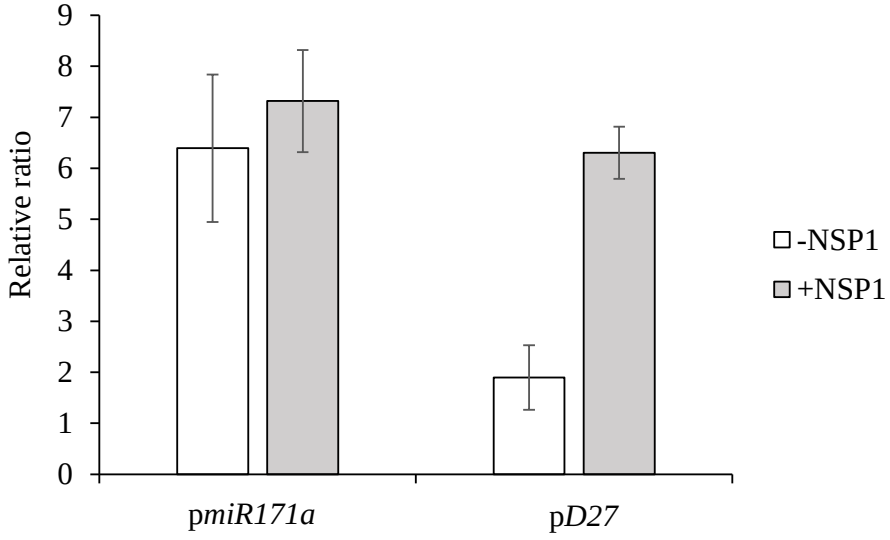


Figure 3: Induction and interaction of *D27* and *MAX1* by NSP1. (A) GUS activity assay of agroinfiltrated tobacco leaves expressing either p*D27*::*GUS* or p*MAX1*::*GUS* in presence or not of *NSP1* or *NSP2*. (B) ChiP-qPCR of the promoter of the miR171a as a negative control and of promoter of *D27*. Both promoters were co-infiltrated with or without NSP1 (- + NSP1) and ChiP was performed with or without the HA antibody against NSP1(HA). The graph represent the relative ratio between the ChiP performed with or without the antibody. Error bars represent the SEM, (A) statistical analysis were conducted using the Krustall-Wallis test, ** represents p value<0.01. (B) Due to the lack of technical repetition (only two) nor statistical analysis could be performed.

We next checked whether NSP1 and NSP2 can transactivate the expression of *D27* and *MAX1*. For this, using the 35S promoter, we expressed *NSP1* and/or *NSP2* in tobacco leaves, together with *D27* or *MAX1* promoter-GUS fusions. Quantification of GUS expression by activity dosage revealed that NSP2 alone is unable to transactivate *D27*/*MAX1* expression, which is coherent with the finding that NSP2 lacks a DNA binding domain (Hirsch *et al.*, 2009), Fig. 3A). In contrast, expression of NSP1, alone or together with NSP2, was sufficient to drive *D27* and *MAX1* expression (Fig. 3A). To know whether NSP1 interacts directly with *D27* and *MAX1* promoters, we performed Chromatin Immuno-Precipitation (ChIP) of NSP1 exhibiting an HA tag (Fig. 3B). As a negative control we used the promoter of the miR171a gene fused to GUS as we previously verified that is was not induced by NSP1 (data not shown). Promoters of both *D27* or *miR171a* were co-infiltrated with or without NSP1 (- + NSP1) and ChiP was performed with or without the HA antibody against NSP1(HA). The graph represents the relative ratio between the ChiP performed with or without the antibody. Our results show that when NSP1 was immunoprecipitated, the amplification of the D27 promoter using qPCR was at least 4 times more abundant than in our controls confirming that NSP1 indeed binds directly to the promoter of *D27* (Fig. 3B). In the case of *MAX1* the results were not convincing enough and the experiment will be repeated.

1.2. Expression analyses of NSP1, NSP2, *D27* and *MAX1* during mycorrhization reveal distinct expression patterns.

Given the importance of strigolactones and of the *NSP1* and *NSP2* genes in the AM symbiosis (Akiyama *et al.*, 2005; Besserer *et al.*, 2006, 2008; Maillet *et al.*, 2011; Delaux *et al.*, 2013), we analyzed whether the above patterns of NSP1, NSP2, *D27* and *MAX1* expression are maintained or modified during AM symbiosis.

We first observed that NSP1 was locally expressed very early in the infection process, in zones corresponding to fungal entry zones, not yet colonized by arbuscules (Fig. 4A). In later symbiotic stages, corresponding to arbuscule-containing tissues, NSP1 expression was no longer visible (Fig. 4B). Surprisingly NSP1 expression was clearly and systematically visible in the vicinity of arbuscule-containing zones but in the not yet well colonized tissues (Fig. 4C, D). NSP1 appears to display a very dynamic and localized expression in the cells that are going to be colonized, and then the expression of NSP1 decreased until a total extinction concomitantly with the colonization of the tissue. This lack of NSP1 expression in the colonized tissues contrasted with the strong expression of NSP2, *D27* and *MAX1* in the same regions (Fig. 5 and Fig. 6 A).

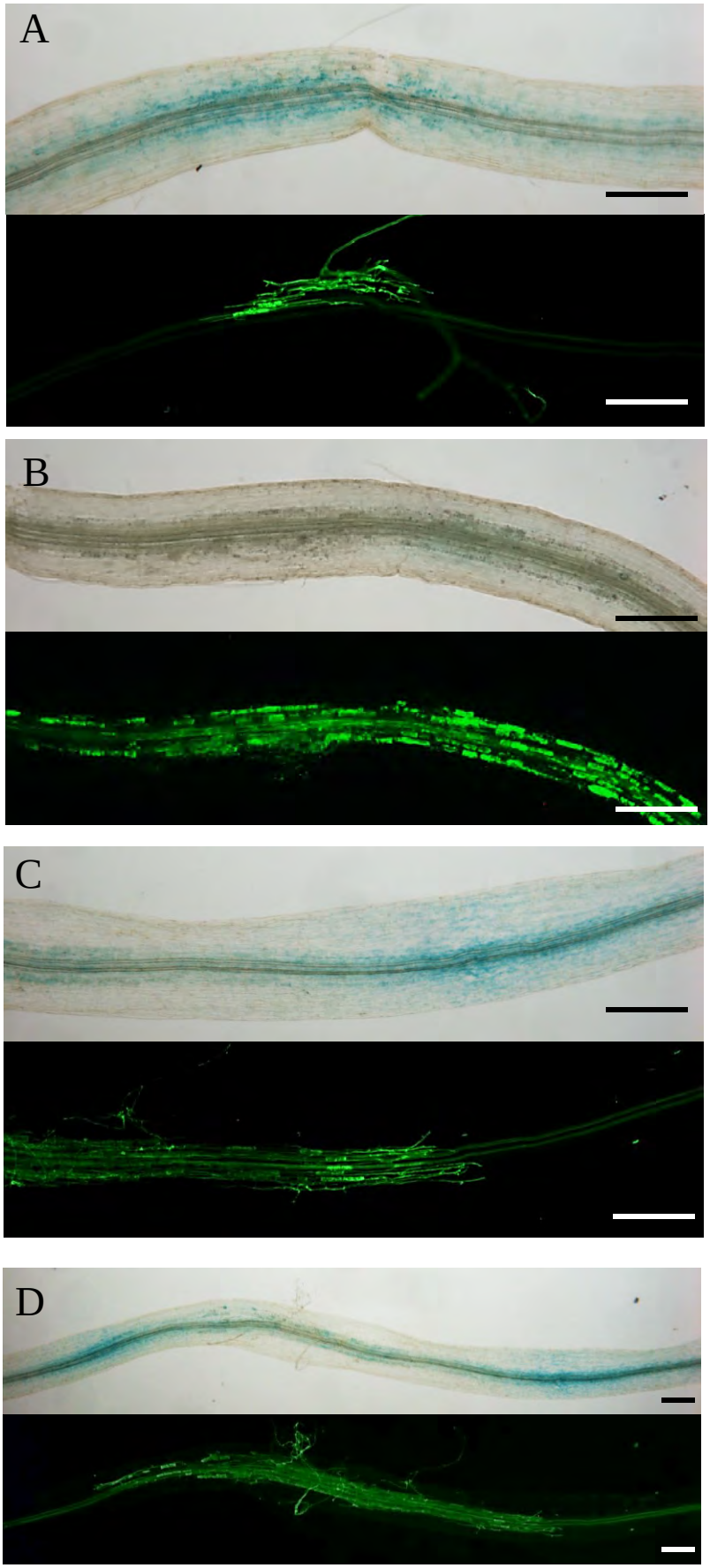


Figure 4:

In contrast with the expression pattern described above in non mycorrhizal root (Figs. 1 and 2), here we did not observe a co-localization of NSP1, *D27* and *MAX1* suggesting that the expression of *D27* and *MAX1* could be regulated by another pathway during mycorrhization. To test this hypothesis we analyzed the expression of *D27* and *MAX1* in mycorrhizal *nsp1* mutant plants and we observed the same pattern as in the wild-type plants: a strong expression of *D27* and *MAX1* in arbuscule-containing tissues (Fig. 6 B).

To further support the hypothesis that an NSP1-independent regulation of *D27* occurs in mycorrhizal roots we assessed the expression of *D27* by qRT-PCR in *M. truncatula* plants colonized by *R. irregularis* at 8, 13 and 27 days post inoculation (Fig. 7). *These experiments were performed in collaboration with Ms. Leonie Luginbuehl from the John Ines center (UK), and will be part of a collaborated work for publication*. In wild-type non-mycorrhizal plants, *D27* expression increased gradually maybe due to a growing phosphate deficiency (López-Ráez *et al.*, 2011, 2014). But in the wild-type, in the 8 day old plants, *D27* expression was higher in mycorrhizal condition compared to non-inoculated plant, confirming that *D27* is induced during mycorrhization. Along time *D27* expression continues to increase as the mycorhization increases and stays about 2 times more expressed (Fig. 7 A red and B). In *nsp1* plants non-inoculated, at the difference of the wild-type, *D27* expression was much lower and remained constant throughout the time periods indicating that this regulation (maybe phosphate induced) was lost in *nsp1* mutant and confirming that NSP1 is crucial for the primary regulation of *D27* expression (Liu *et al.*, 2011). But interestingly, in mycorrhized conditions the expression pattern of *D27* whose expression increases throughout the three time periods is similar in mycorrhizal wild-type and *nsp1* plants. This up regulation, correlated with the mycorrhizal state of the plants and presumably localized in the colonized regions (Fig. 7 A, B), is very subtle but significant. A similar experiment was done on MAX1 gene but without revealing similar pattern in the *nsp1* mutant, suggesting the absence or the less strong activity of a NSP1 independent induction.

Figure 4: Expression pattern of translational fusion of *NSP1* in *Medicago* A17 WT hairy roots during the different steps of mycorrhization. (A) NSP1 is expressed very early upon fungal entrance before arbuscule formation. (B) NSP1 is not expressed or do not accumulate in the arbuscule containing cells. (C-D) NSP1 is expressed in the cells located just before the fungal internal hyphae arrival. Upper pictures show the bright field images, the bottoms ones are the respective images under fluorescent light showing the fungus stained with WGA-FITC. Scales 200µm.

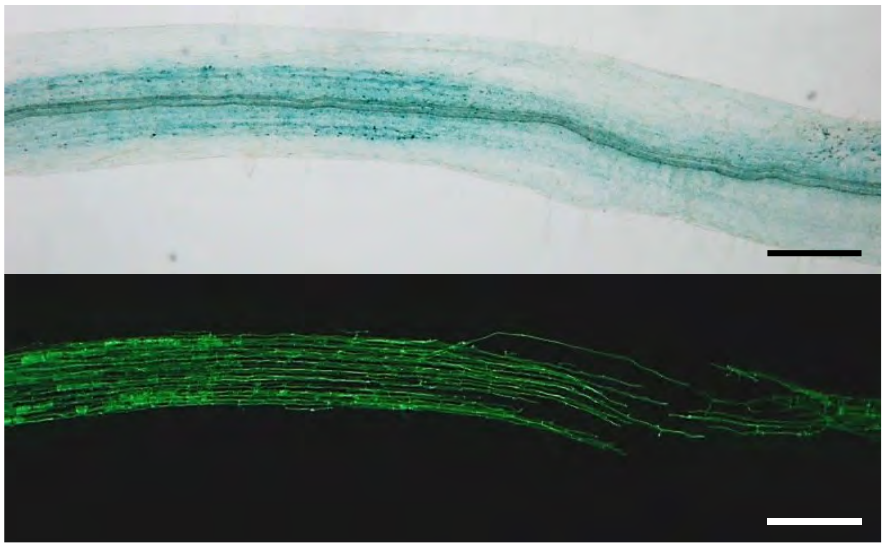


Figure 5: MtNSP2 is expressed in fungal containing tissues. Expression pattern of translational fusion of *NSP2* in *M. truncatula* WT hairy roots during mycorrhization. Upper pictures show the bright field images, the bottoms ones show the fungus stained with WGA-FITC. Scales =200µm.

Altogether, these data confirm that NSP1 plays a key role to regulate *D27* and *MAX1* expression but they also suggest that it is not involved in the specific regulation of *D27* and *MAX1* expression in the colonized tissues of mycorrhizal roots.

1.3. NSP1 and NSP2 play different roles during mycorrhization.

To go further in the understanding of the role of both NSP1 and NSP2 during AM symbiosis, we performed a detailed phenotyping of the first steps of mycorrhization, from penetration of the fungus to arbuscule formation. We observed a much lower number of infection sites in the *nsp1* mutant when compared to the wild-type. This defect of fungal penetration was not observed in the *nsp2* mutant (Fig. 8 A). It was then from partially to completely complemented by treatment with the synthetic strigolactone analogue GR24 (in our different repeats), strongly suggesting that the inability of *nsp1* mutant to synthesize strigolactones (Liu *et al.* 2011) was partly responsible for this mycorrhizal phenotype (Fig. 9 A). The addition of GR24 would have compensated for the absence of strigolactones exuded in the rhizosphere and allowed pre-symbiotic stimulation of the fungus. The phenotype of the double mutant presented a similar reduction of fungal entrance, consistent with the single mutant *nsp1* (Fig. 7 A).

We then phenotyped later stages of mycorrhizal colonization by quantifying, for each infection points, the extent of fungal propagation in the root and the related arbuscule density. The accurate analysis of arbuscule density for each fungal entrance (ranked from 1, no arbuscule, to 4 many arbuscules, Scale picture in Fig. S2) showed that in the *nsp1* mutant the abundance of arbuscules was strongly reduced (Fig. 7 C), suggesting that NSP1 plays a positive role in arbuscule effective formation. These results are also confirmed by a similar work but another quantification method performed at the John Ines Center by Leonie Luginbuehl (Fig. 7 B). These results are not in agreement with the phenotype presented in Delaux et al., 2013 showing that *nsp1* did not display an arbuscular phenotype. However, because we look at very early stages at each infection points, we could unravel subtle phenotypes that could be hidden during the long term mycorrhization experiment (8 weeks), especially when several fungal entrances are happening in the same root portion.

When we pursued our phenotyping analysis we surprisingly saw that the average length of colonization from each infection site was higher in the *nsp1* mutant than in the wild-type, suggesting that if NSP1 plays a positive role in arbuscule formation it plays a negative role in

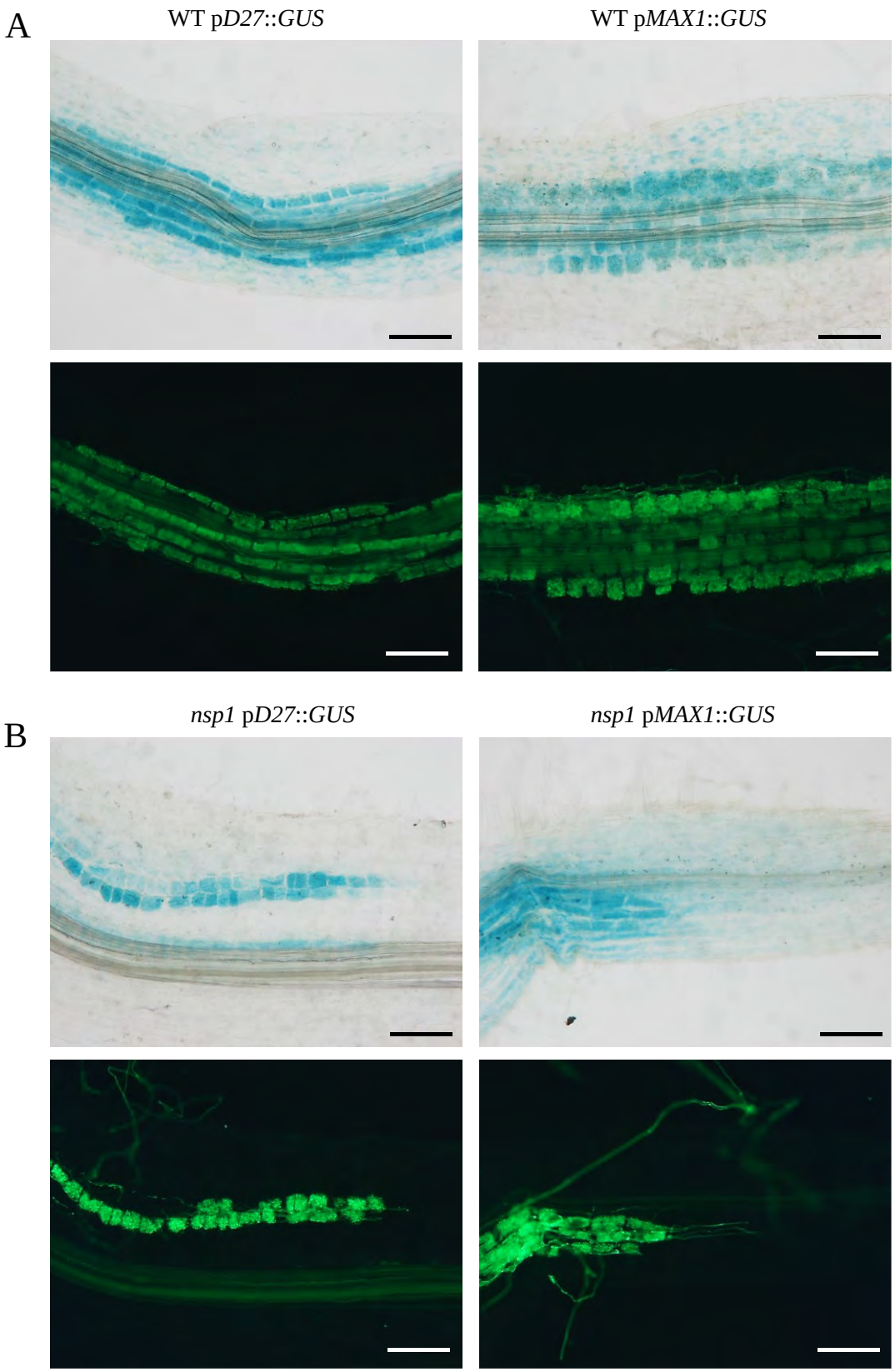


Figure 6: *MtD27* and *MtMAX1* are expressed in fungal containing tissues. Expression pattern of transcriptional fusion of *D27* and *MAX1* promoter in Medicago WT (A) or *nsp1* (B) hairy roots during mycorrhization. In all condition both *D27* and *MAX1* promoter seems to be expressed in the arbuscule containing tissues. Upper pictures show the bright field images, the bottoms ones show the fungus stained with WGA-FITC. Scales =100µm.

hyphal propagation within the root (Fig. 8 B). To determine if the lack of strigolactones in the *nsp1* mutant was responsible for these phenotypes we treated mycorrhizal mutant and wild-type plants with GR24. Figures 9 B and C reveal that GR24 treatments of the *nsp1* mutant do not stimulate arbuscule formation or reduce intraradical hyphal extension of the fungus, *i.e.* do not restore the wild-type phenotypes. These results suggest that either exogenous application of GR24 cannot reach and regulate the fungus when growing in the root, or the intraradical hyphal extension as well as the arbuscule formation are not strigolactone dependent.

We then performed the same phenotyping analysis of the *nsp2* plants and in contrast with what we observed in the *nsp1* mutant, hyphal propagation inside the roots of *nsp2* was reduced compared to the wild-type (Fig. 8 B). But similarly to what we found in *nsp1* roots, arbuscule abundance was also reduced (although to a lesser extent) in *nsp2* roots confirming previous report (Maillet *et al.*, 2011) (Fig. 8 C). In roots of the double *nsp1nsp2* mutant hyphal propagation was like in the *nsp1* mutant, as if this trait was "dominant", and arbuscule abundance was intermediate between those found in the single mutants. Altogether these results highlight a complex interplay between the NSP1, NSP2 and various regulations of the mycorrhization process, with one that concerns only NSP1 (fungal penetration), a second one (intraradical hyphal propagation) and a third one (arbuscule formation) that are antagonistic and synergistic between NSP1 and NSP2, respectively.

2. Discussion:

We have accumulated several experimental evidences suggesting the different and multiple implications of the GRAS transcription factors NSP1 and NSP2 in potentially root development and mycorrhization.

We have confirmed the implication of NSP1 in the regulation of the two SL biosynthesis genes *D27* and *MAX1* (Liu *et al.*, 2011). This regulation seems to occur by direct interaction of NSP1 with *D27* promoters (and potentially *MAX1* promoters), mainly in root primordia and meristematic/elongation zones (Fig. 1 and 2). At these stages of root development NSP1 appears to be crucial for the induction of *D27* and *MAX1* since their expression is abolished in the *nsp1* mutant (Fig. 2). This very localized expression, if it is correlated to the synthesis of SLs, might be related to the action of SLs as modulator of auxin distribution and especially repolarization of PIN auxin transporters (Crawford *et al.*, 2010; Shinohara *et al.*, 2013).

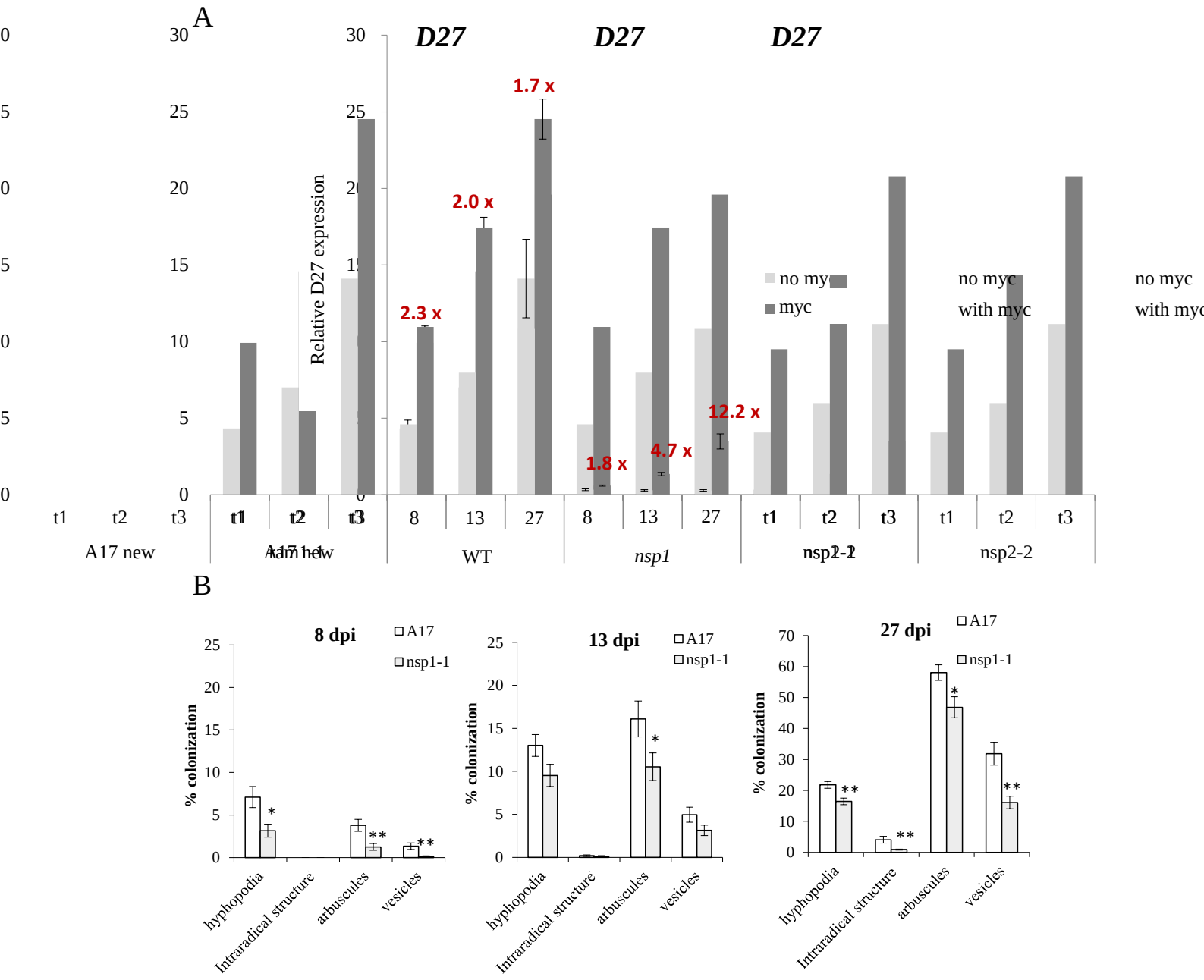


Figure 7: D27 expression measured by qRT-PCR in a time course mycorrhization assay in *M. truncatula* WT and in the *nsp1* mutant. (A) qRT-PCR experiment showing D27 expression in *M. truncatula* WT and in the *nsp1* mutant. Both WT and the *nsp1* mutant were inoculated with *R. irregularis* at t0 (dark grey), while same amount of plant were not inoculated (light grey). In abscise is represented the number of days post inoculation. In red are represented the relative induction between the non-inoculated conditions compared to the inoculated conditions. (B) Mycorrhizal phenotype of the corresponding time conditions used for the qRT-PCR. Error bars represent SEM. (A) For both genotypes the difference in expression between "no myc" and "myc" is statistically significant from $p<0.05$ to $p<0.01$, (not shown). Standard t-test was made for each conditions. Pvalue *<0.05, **<0.01. *These experiments were realized in collaboration with Leonie Luginbuehl from the John-Ines center (UK).*

As SLs have also been proved to negatively influence lateral root priming and emergence, it is possible that the three genes *NSP1*, *D27* et *MAX1* play an important role in root architecture (Koltai *et al.*, 2010b; Kohlen *et al.*, 2011; Liu *et al.*, 2013; Jiang *et al.*, 2016). Furthermore, NSP1, in addition to its roles in SL biosynthesis, might intervene in root cell reprogramming via the transcriptional regulation of other genes, or via its interaction with other GRAS transcriptional factors crucial for root development.

In the presence of an AM fungus, *NSP1* induction, and consequently that of *D27* and *MAX1,* could be triggered by Myc-LCOs (Delaux *et al.*, 2013; Camps *et al.*, 2015) (Fig. S3). In a positive feedback loop, we can speculate that this induction, during the early process of fungal infection, will stimulate the pre-symbiotic growth of the fungus, as a result of an increased SL production/exudation. In agreement with this pivotal role of NSP1, early in the mycorrhization process, is the fact that the mutation of its encoding gene leads to a much fewer infection sites (Fig. 8 A). Myc-LCO are also known to stimulate lateral root formation (perhaps via *NSP1* induction) (Maillet *et al.*, 2011; Sun *et al.*, 2015b,a; Tanaka *et al.*, 2015). If these lateral roots, where *D27* and *MAX1* are mainly induced (Fig. 2), are privileged sites for SL production/exudation, it is not surprising that they are also root sites more suitable for fungal colonization.

However, later during the mycorrhization process, this direct role of NSP1 on SL biosynthesis seems to be different. Whereas both *D27* and *MAX1* are expressed in the arbuscule containing tissues, NSP1 is not, strongly suggesting that the expression of *D27* and *MAX1* is not NSP1 dependent in these regions (Fig. 4 and 6 B). This indicates that during mycorrhization the control of SL biosynthesis is supported by another regulatory pathway, independent of NSP1. The hypothesis of the regulation of *D27* by an NSP1-independent manner has been already suggested in Lotus (Nagae *et al.*, 2014) and pea (Shtark *et al.*, 2016), and here we confirm this hypothesis by providing additional spatiotemporal clues. Moreover, transcriptomic approaches in *Medicago* have also highlighted that the induction of *D27* by exogenous Myc-LCOs treatments was not totally NSP1-dependent, as *D27* was still induced by non-sulfated Myc-LCOs in *nsp1* and *dmi3* mutants (Hohnjec *et al.*, 2015). Hence a component of the *D27* regulation pathway in mycorrhizal conditions seems to be independent of the canonical common symbiotic signaling pathway. It may concern a large set of genes and functions since a high number of genes were found to be differentially expressed in

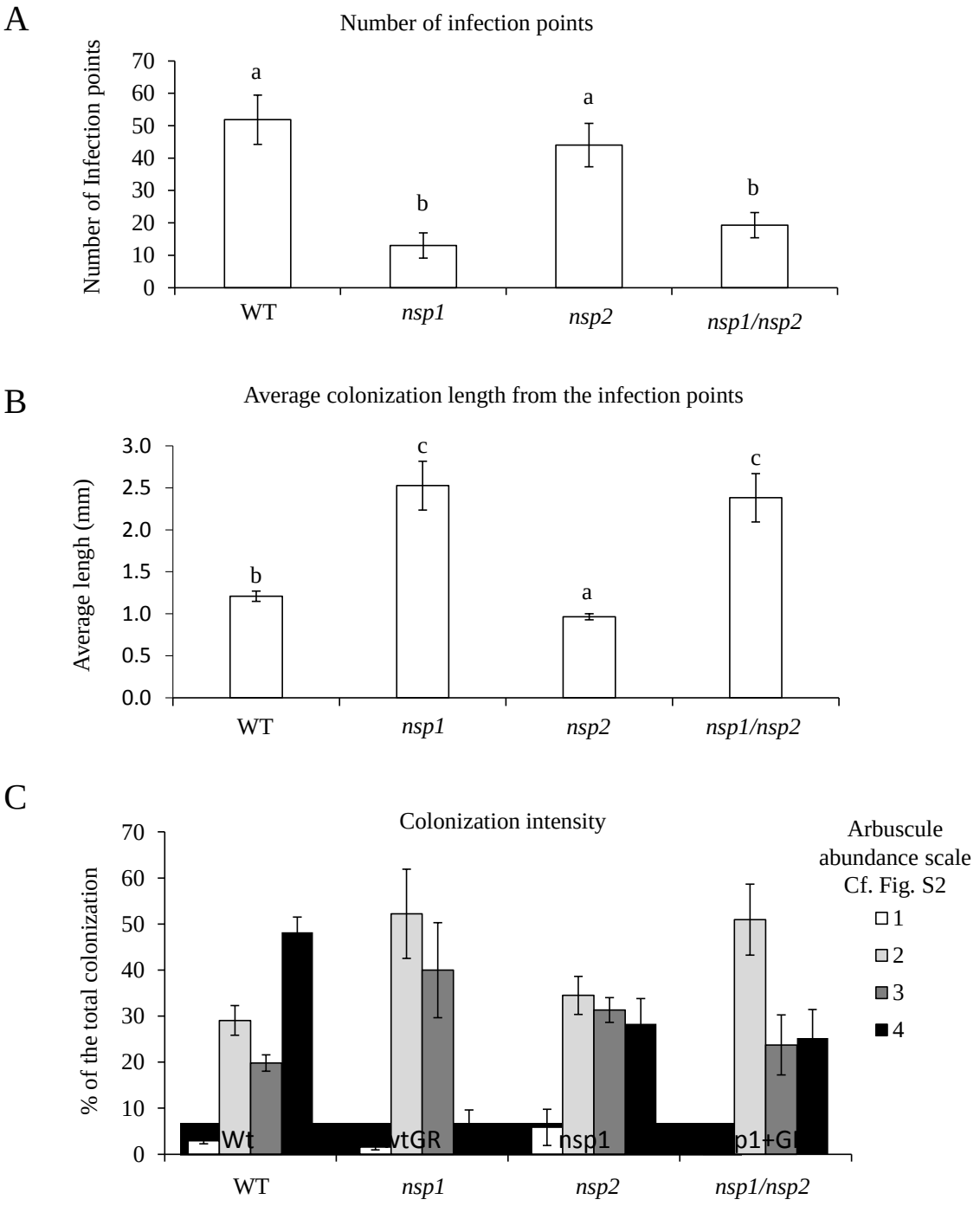


Figure 8: Mycorrhizal phenotyping of two weeks old *M. truncatula* plant, WT, *nsp1*, *nsp2* and the double mutant *nsp1/nsp2*. (A) Measure of the average number of infection point per plant from the different genotypes. (B) For each infection points the distance of colonization has been measured, the graph represents the mean colonization length for the different genotypes. (C) The abundance of arbuscule has been evaluated for each infection point (Scale on Fig. S2), the graph represents the proportion of the total colonization having the different abundance. Error bars represent the SEM, (n=10). Significance levels are based the Krustall-Wallis test (A) and on Tukey's post-test (1-way ANOVA), (B). a-c represent a pvalue <0.05.

M. truncatula, in an NSP1 independent manner, following Myc-LCO treatment (Camps *et al.*, 2015; Hohnjec *et al.*, 2015).

Once root colonization by the fungus is well established, NSP1 seems to have other regulatory roles, not related to SLs. Indeed, NSP1 proteins seem to accumulate preferentially in not yet colonized tissues just ahead of the colonization front, while their presence seems to strongly diminish in the colonized sections. This localization pattern is opposite to that of *D27* and *MAX1* whose expression is confined to the highly colonized zones. We can speculate that the expression of NSP1 in these specific, not yet colonized, mycorrhizal zones could be to slow down the hyphal progression and prepare root cells for arbuscular colonization (Camps *et al.*, 2015). This "priming" could be necessary for proper arbuscule development (Genre et al. 2008). However, NSP1 does not appear to be essential for arbuscule morphogenesis but more for an optimal fungal colonization.

By using similar approaches we highlighted that the expression pattern of NSP2 and its functions were clearly different to those of NSP1 during mycorrhization. Contrary to *nsp1* plants that were less often infected by the fungus, had longer intraradical hyphal extension and reduced arbuscule formation when compared to the wild-type, the *nsp2* plants had also a reduced arbuscule formation but a normal number of infection sites and a reduced intraradical hyphal extension. As shown by Liu et al. (2011), unlike *nsp1* plants that do not produce detectable amounts of SLs, *nsp2* plants over-accumulate didehydro-orobanchol. This compound recently identified as medicaol, like other SLs (Akiyama *et al.*, 2010), has been shown to stimulate growth of AM fungi (Tokunaga *et al.*, 2015). It is then not surprising that fungal entry did not seem to be disturbed in this mutant since the plant is still able to produce (and probably exude) a stimulatory SL in the rhizosphere (Fig. 8 A). Taking into account that NSP2 does not possess any DNA binding domain (Hirsch *et al.*, 2009), its action as a TF requests an interaction with other TFs. In agreement with this, NSP2 has been shown to interact with several other GRAS TFs which are involved in the mycorrhization process, like RAD1 or TF80 (Park *et al.*, 2015). NSP2 also interacts with RAM1, itself interacting with other GRAS TFs like DIP1 which regulates DELLA (Gutjahr, 2014; Park *et al.*, 2015). NSP2 could then play multiple roles improving the efficiency of several TFs for the regulation of a large set of target genes (Cerri *et al.*, 2012). Given the fact that NSP2 is present in the colonized tissues we can speculate that an *nsp2* mutation might perturb hyphal propagation in the root and arbuscule formation. Finally, *NSP2* expression is under the control of the miR171h that is also expressed in the arbuscule containing regions

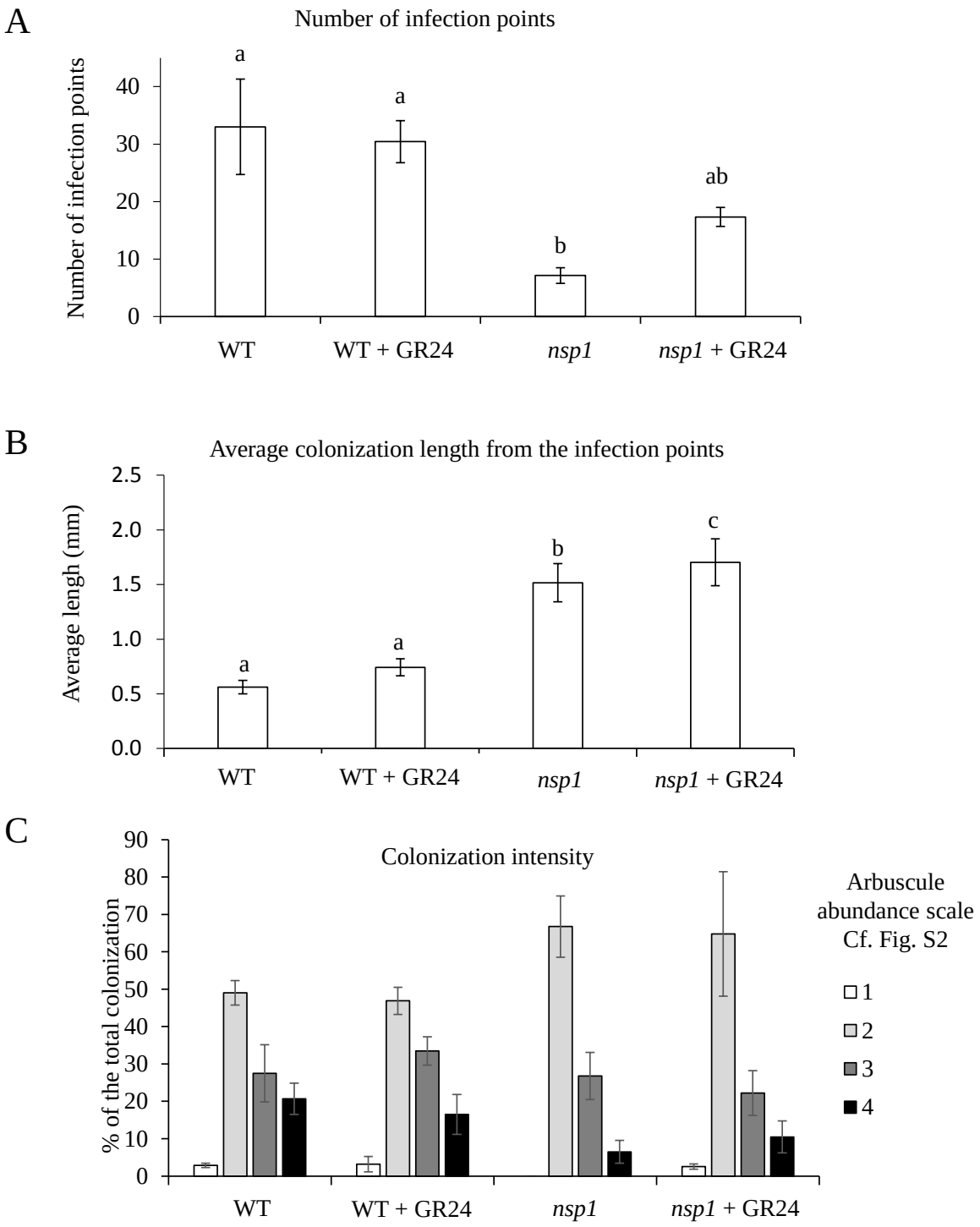


Figure 9: Mycorrhizal phenotyping of two weeks old *M. truncatula* plant, WT and the *nsp1* mutant treated or not with GR24. (A) Measure of the average number of infection point per plant from the different treatments. (B) For each infection points the distance of colonization has been measured, the graph represents the mean colonization length for the different treatments. (C) The abundance of arbuscule has been evaluated for each infection point (Scale on Fig. S2), the graph represents the proportion of the total colonization having the different abundance. Error bars represent the SEM, (n=10). Significance levels are based the Krustall-Wallis test (A) and on Tukey's post-test (1-way ANOVA), (B). a-c represent a pvalue<0.05.

(Lauressergues *et al.*, 2012; Hofferek *et al.*, 2014), adding an additional complexity layer to the action and regulation of this transcriptional factor.

It is still not really clear whether or not NSP2 is indispensable for *D27* and *MAX1* induction, during both the asymbiotic and mycorrhizal conditions but several experiments are planned to uncover this veil. However, as shown by Liu et al., 2011 it is highly probable that NSP2 is involved in the regulation of *D27* and *MAX1* expression at least in asymbiotic conditions.

Finally the question of the role of SLs in planta, after the fungal entrance in the root is still open. Our data suggest that both *D27* and *MAX1* are expressed in the arbuscule-containing tissues via an NSP1 independent induction, but there is no evidence that SLs are involved in the later steps of mycorrhization. However, the SL perception by the plant could have an influence on AM colonization since a rice SL insensitive mutant *d3* shows an incapacity to sustain arbuscule development (Yoshida *et al.*, 2012). In addition, some experiments point to the regulation of SL production in roots according to their mycorrhizal status. Indeed after a certain colonization threshold, the SL production in roots has been shown to decrease (López-Ráez *et al.*, 2011, 2014). This would be the result of some autoregulation mechanisms that temper additional fungal entrances. As SL biosynthesis is also controlled by phosphate, the reduction of SL content in mycorrhizal roots could also simply be the result of a higher phosphate nutrition.

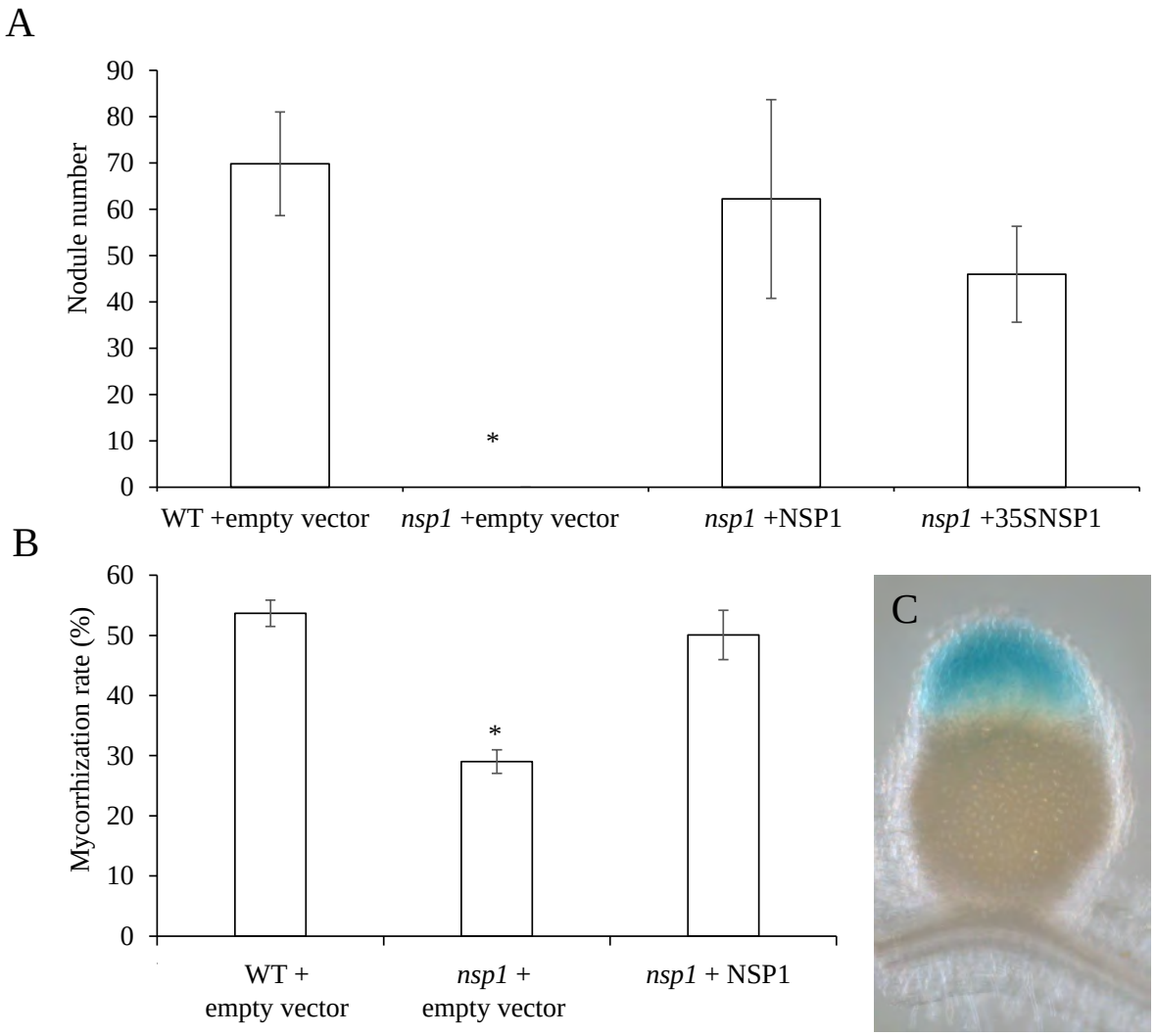


Figure S1: Expression of p*NSP1*::*NSP1*::*GUS*::UTR in chimeric *nsp1 Medicago* plants is able to restore the defect in nodulation (A) and mycorrhization (B). (C) Stereomicroscopic image of a nodule from a complemented *nsp1* mutant. GUS expression pattern shows a very specific expression of NSP1 in the meristematic zone I and II of the nodule. Error bars represent the SEM. Both experiment were repeated two times, statistical analysis were conducted using the Krustall-Wallis test. (A) n=5 (B) n=8, * represent a pvalue<0.05)

3. Material and Methods:

3.1. Biological material:

Seeds of *M. truncatula* Gaertn 'Jemalong' genotype A17, *nsp1-1* (Catoira et al., 2000; Smit, 2005), *nsp2-2* (Oldroyd, 2003; Kalo, 2005) and *nsp1/nsp2* (provided by Geurts R., Liu et al., 2011) were scarified by incubation in concentrated (98%) H_2SO_4 for 8 minutes. Then they were surface-sterilized using 9% NaClO for one minute before to be washed with sterile water and germinated on agar plates in the dark for 5 days at 4°C. For GUS expression analysis chimeric plants with transformed roots (see below) were cultivated in 250 mL pots (one chimeric plant per pot) filled with Oil-Dri US-special substrate (Damolin, www.damolin.fr) for 5 weeks in a growth chamber (16/8 h day/night, 24°C/22°C, 120-150 µmol $m^{-2}s^{-1}$), and watered every 2 days with modified Long Ashton medium containing a low concentration of phosphate (7.5 µM) (Balzergue *et al.*, 2011).

Nodulation assays were performed by infecting plants with the *Sinorhizobium meliloti* Sm2011 strain, constitutively expressing the YFP (from P. Smit, provided by J. Fournier, LIPM, Toulouse, FR) and pre-cultivated on standard TY medium with 10 µgml^{-1} tetracycline and 6 mM of $CaCl_2$, at 28°C. Plants were inoculated with a suspension of bacteria centrifuged (10 min at 4000rpm) and resuspended in water (DO_{600}=0.05, 10 ml per pot) and harvested two weeks post infection.

For mycorrhization experiments, plants were inoculated with *Rhizophagus irregularis* DAOM 197198 sterile spores (2000 spores per liter of substrate) purchased from Agronutrition (Carbone, France).

The mycorrhizal phenotyping experiments were done by growing the seedlings in 50 ml Falcon tubes from which the conic bottom part was cut. Tubes were used upside-down (with the lid at the bottom) and 50 spores of *Rhizophagus irregularis* were added in the lid before filling the tube with Oil-Dri substrate. Germinated seedlings were planted in the tubes (one seedling per tube), grown for 2 weeks in a growth chamber (16/8 h day/night, 25°C/23°C, 260-300 µmol $m^{-2}s^{-1}$) and watered every two days with 4 ml of modified Long Ashton medium containing a low concentration of phosphate (7.5 µM, Balzergue *et al.*, 2011).

Nicotiana benthamiana plants were grown in standard compost with perlite (4:1), in a growth chamber (16/8 h day/night, 24°C /22°C, 200-220 µmol $m^{-2}s^{-1}$) for 5 to 6 weeks (plant display typically at least 5-6 leaves).

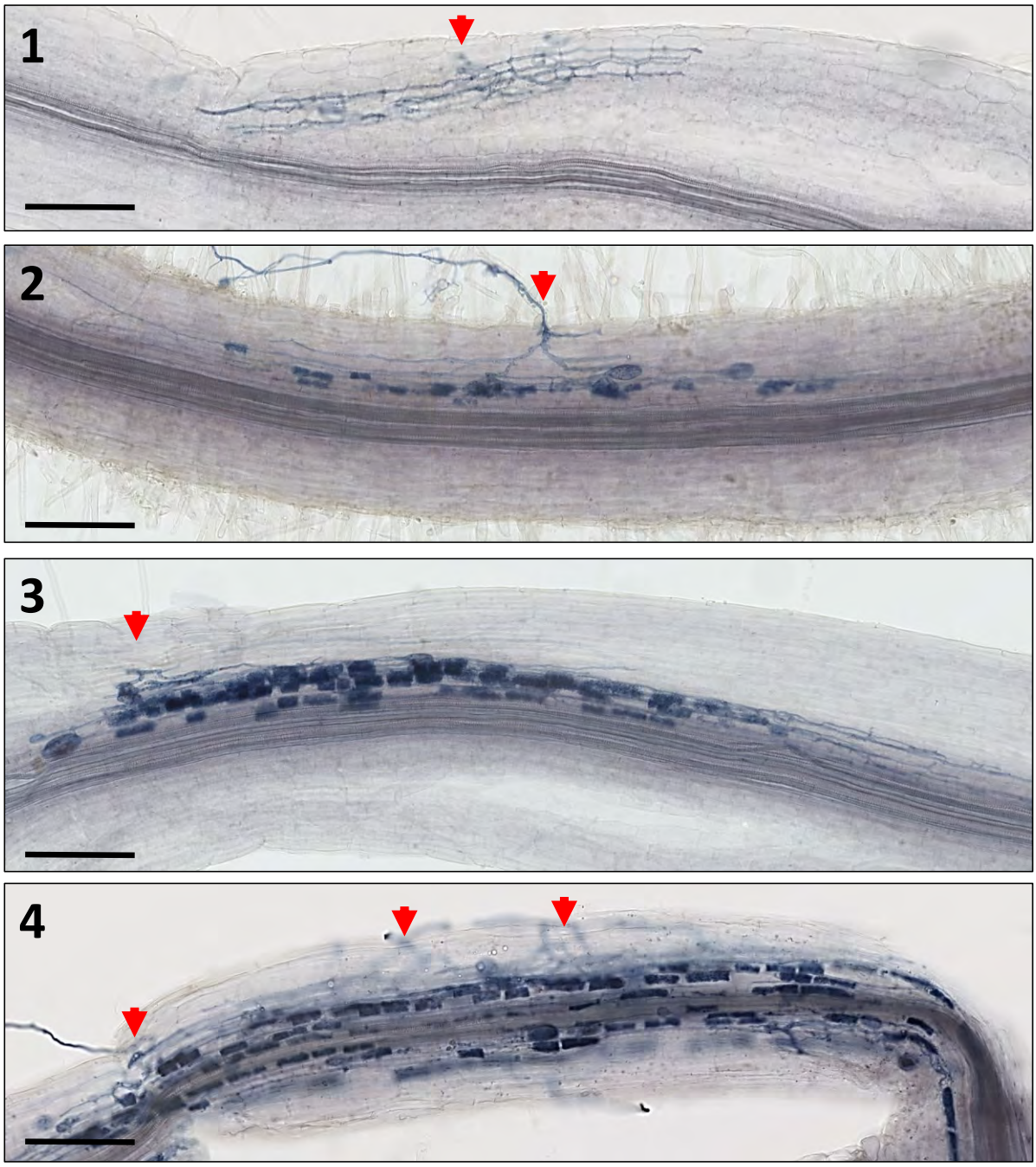


Figure S2: Mycorrhization scale used to evaluate the arbuscule abundance in Fig. 8C and Fig. 9C. Number on the left represent the scale value, in red are shown the fungal infection points. Scale 200µm.

3.2. Genes used and plasmid construction:

Genes used in this studies were *Mt-NSP1* (Medtr8g020840), *Mt-NSP2* (Medtr3g072710), *Mt-D27* (Medtr1g083360) and *Mt-MAX1* (Medtr3g104560).

A modified pCAMBIA2200 binary vector was used with the Golden Gate strategy for cloning (Engler *et al.*, 2008). The DNA fragments of interest from *M. truncatula* were flanked by BsaI restriction sites during the PCR amplification step using Pfu polymerase (Promega, www.promega.com), primers shown in Table S1. One-step digestion–ligation reactions were carried out with 100 ng modified pCAMBIA, 100 ng of each PCR fragment, 1 µl 10× ligase buffer (Promega), 2.5 U T4 DNA ligase (Promega), 2.5 U BsaI (NEB), in a final volume of 10 µl. Incubation was performed at 37 °C for 30 min and 16 °C for 30 min and repeated once. A final incubation step at 50 °C for 20 min was used to cleave any remaining undigested cloning vector. The amplified promoters were p*NSP1* (3183 bp), *NSP1* post CDS section (3180 bp), p*NSP2* (2890 bp), p*D27* (2918 bp for tobacco agroinfiltration or 1045 bp for root transformation) and p*MAX1* (3061 bp for tobacco agroinfiltration and 928 bp for root transformation). All primers used are listed in Table S1. pCAMBIA carries a kanamycin resistance (25 µg/ml) and DsRED protein expression in Agrobacterium and in plants.

3.3. Tobacco agroinfiltration and GUS quantification:

Nicotiana tabacum leaves were agroinfiltrated following the protocols (Yang *et al.*, 2000). Forty hours after infiltration, total proteins were extracted from 100 mg of transformed leaves after grinding in liquid nitrogen with 100 µl of GUS buffer (100 mM Phosphate buffer pH 7, 0.1% TritonX-100, 10 mM β-mercaptoethanol) (Wagner *et al.*, 2015). Glucuronidase activity was measured by fluorometric assay with 25 µl of protein extracts and 1 mM MUG (4-methylumbelliferyl glucuronide, Sigma) in a total reaction volume of 200 µl. Fluorescence was measured every 5 min during 120 min on a TriStar LB 941 Multimode Microplate Reader (Berthold Technologies) at 37°C with 360 nm excitation and 460 nm emission. The fluorimeter was calibrated with freshly prepared MU4 (4-methylumbelliferone sodium salt, Sigma-Aldrich) standards in the same GUS buffer. Normalization was done by measuring the total protein concentration by the Bradford method on 96 well plates. Two hundred microliters of Bradford reagent (Bio-Rad Laboratories) were added to 5 µl of samples. After incubation (15 min, 25°C), absorbance was measured at 565 nm. Standard curve was done with 1–20 µg of BSA (Sigma-Aldrich). Glucuronidase activity was calculated from the linear part of the reaction (between 20 and 100 min) and expressed as nkatal/mg of total proteins.

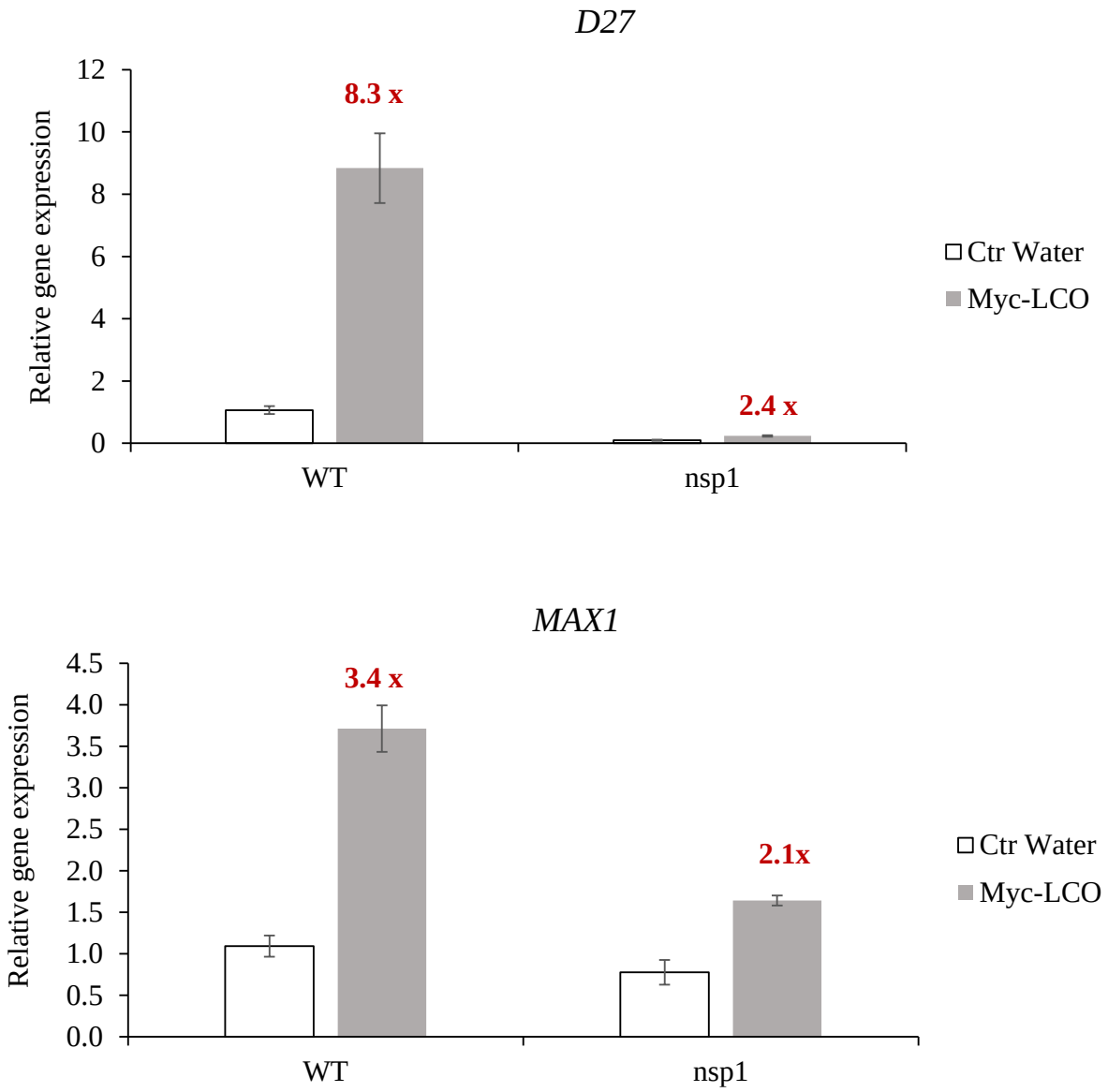


Figure S3: Induction of *D27* and *MAX1* in response to Myc-LCO is partially NSP1 independent. Relative gene expression of *MtD27* and *MtMAX1*, in either the WT plant or the *nsp1* mutant, measured by qRT-PCR, treated with either water or by Myc-LCO (10^{-7}M) during 10h. In red is shown the relative induction between the control and the Myc-LCO treatment. Errors bars represent SEM.

3.4. Root transformation:

As described by Boisson-Dernier et al. (2001), two days before transformation, *Agrobacterium rhizogenes* ARqua1 (Quandt, 1993) cultures containing the constructs of interest were grown on solid LB supplemented with the antibiotic of selection kanamycin (25 µg/ml) at 28°C.

In a Petri dish with sterile water, approximately 1 cm of root tip of each five-day-old seedling was removed. Wounded root tips were then dipped in the bacterial layer, and immediately transferred on 12 cm square plates filled with Farhaeus medium gelled with Bacto™ Agar, Becton, Dickinsson and Company, Sparks, MD, USA (7%) and supplemented with the selective antibiotic kanamycin (25 µg/ml). The plates were incubated in a 24°C, 16 h/8 h day/night, 60 µmol $m^{-2}s^{-1}$ growth chamber for 3 weeks. Seedlings were then screened with a stereomicroscope Axio Zoom.V16 (Zeiss, Oberkochen, Germany) under RFP Plus filter set (Uex=546/12; Uem=607/80), to discard all the out-growing, non-DsRED expressing, wild-type roots. Before fungal inoculation, the selected chimeric plants were acclimated in Oil-dri US special substrate for one week under saturated hygrometry.

3.5. Strigolactone treatments and Myc-LCO treatment:

The SL analog racemic GR24 was purchased from Chiralix B.V. (Nijmegen, The Netherlands). For supplementation of mycorrhizal seedlings with GR24, 10^{-8} M solution was used in low phosphate Long Ashton and watered (4 ml/Falcon) three times a week. Control plants were watered with 0.0001% (v/v) acetone.

For Myc-LCOs treatments, 7 day old *Medicago* seedling grown in vitro (see above) were treated with 5ml of 10^{-7}M of the LCO-IV(C16:0,S) Myc-LCO for 10h before collecting the samples. Myc-LCO was described by Maillet *et al.* (2011) and provided by Eric Samain, Sébastien Fort and Sylvain Cottaz (CERMAV, Grenoble, France).

3.6. Gene expression analyses*:*

For quantitative RT-PCR analyses, total RNA was extracted using a Plant RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated by DNase I (Promega) to remove genomic DNA contamination. Reverse transcription was performed using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) on 1 µg of total plant RNA. For each experiment, six to twelve independent plants were analyzed. Quantitative PCR amplifications were conducted on a Roche LightCycler 480 System (Roche Diagnostics) under the following conditions: 95°C for 5 min, then 45 cycles of 95°C for 15 sec and 60°C for 1 min.

Primer name	Sequence 5'-3'
Golden-Gate cloning	
GoldGate MtNSP1 prom+gene Fw BsaI	AAAGGTCTCAAAATATGCCTTTATCATTTTTGGGG
GoldGate MtNSP1 prom+gene Rv BsaI	AAAGGTCTCACCATTTCTGGTTGTTTATCCAGTTTCC
GoldGate MtNSP1 3'UTR Fw BsaI	AAAGGTCTCCTTCGTTCGAGCTCAACATTGACAGCA
GoldGate MtNSP1 3'UTR Rv BsaI	AAAGGTCTCCCGTACCTCCTCCTTATACTTTCTTG
GoldGate MtNSP2 prom+gene Fw BsaI	AAAGGTCTCCAAATTGTAGACTTCAATAAACTAAT
GoldGate MtNSP2 prom+gene Rv BsaI	AAAGGTCTCTCCATTAAATCAGAATCTGAAGAAGAAC
GoldGate MtNSP2 3'UTR Fw BsaI	AAAGGTCTCCTTCGGATGCTGATTAATTAAGTGCTAA
GoldGate MtNSP2 3'UTR Rv BsaI	AAAGGTCTCCCGTAGTCCTTAAACCTACACTTTAG
GoldGate MtD27 prom 3kb Fw BsaI	AAAGGTCTCCAAATCAGGTAAACCCCCCTTTTC
GoldGate MtD27 prom 1kb Fw BsaI	AAAGGTCTCCAAATAGATTCACACATTATTTTACG
GoldGate MtD27 prom Rv BsaI	AAAGGTCTCTCCATTTGTGAGTAGTTATTGATTTTCAT
GoldGate MtMAX1 prom 3kb Fw BsaI	AAAGGTCTCAAAATTTTAGAAAAGTTGATCCGGATC
GoldGate MtMAX1 prom 1kb Fw BsaI	AAAGGTCTCAAAATGACCTCAAAAAACCATTTTTTTTA
GoldGate MtMAX1 prom Rv BsaI	AAAGGTCTCACCATTGCAAATTCAATAGAGTAGAGA
GoldGate MtNSP2 prom Rv BsaI	AAAGGTCTCTCCATGGTATAATTAAGTTAGGT
GoldGate MtNSP1 prom Rv BsaI	AAAGGTCTCTCCATTGTAATGAAAAAACAGAAAAAA
GoldGate MtNSP2 gene+UTR Fw BsaI	AAAGGTCTCCAATGGATTTGATGGACATGGATG
GoldGate MtD27 RNAi sens Fw for 35S prom BsaI	AAAGGTCTCCTAGCATGGATTCAAAGATGATTGCAC
GoldGate MtD27 RNAi sens Fw for vapy prom BsaI	AAAGGTCTCCGTGTATGGATTCAAAGATGATTGCAC
GoldGate Prom vapy golden Fw BsaI	AAAGGTCTCcAAATCGGTAAGGGTTACATAAAAGAT
GoldGate MtD27 RNAi sens Rv BsaI	AAAGGTCTCCGCGTCTGTTGCTGCTTGAACACTTT
GoldGate MtD27 RNAi antisens Fw BsaI	AAAGGTCTCCATTACTGTTGCTGCTTGAACACTTT
GoldGate MtD27 RNAi antisens Rv BsaI	AAAGGTCTCCCGTAATGGATTCAAAGATGATTGCAC
qRT-PCR	
MtUbiquitin qpcr Fw	GCAGATAGACACGCTGGGA
MtUbiquitin qpcr Rv	AACTCTTGGGCAGGCAATAA
MtD27 qpcr Fw	GAGATGATATTCGGCCAGGA
MtD27 qpcr Rv	GTTGCTTGAGTGCTGGATCA
MtMAX1qpcr Fw	TTAAACTCGCGACTGACGTG
MtMAX1 qpcr Rv	TTTCGTTGTGAACGGAATGA

Table S1: Primers used for Golden-Gate cloning, and qRT-PCR

The measured transcripts were normalized by using the *Mt-Ubiquitin* gene. The primers used in this study are listed Table S1.

For histochemical GUS analysis, root tissues of the different GUS expressing chimeric plants were first fixed in 1% formaldehyde in 100 mM sodium phosphate buffer, pH 7.2, under vacuum for 15 min and then soaked in GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1 % Triton X-100, 0.3 mg ml^{-1} X-Gluc) under vacuum for 15 min. Root tissues were then incubated from 30 min to 24 h in GUS staining solution at 37 °C.

Any subsequent fungal staining using WGA-FITC was performed on GUS stained roots, cleared with KOH and stained with WGA-FITC as described below for mycorrhizal phenotyping.

3.7. Chromatin Immuno-Precipitation.

Tobacco leaves were transformed as described above to express in this heterologous system *Mt*p*D27* or *Mt*p*MAX1*::*GUS* constructs with or without the co-expression of the 35S::*MtNSP1*(HAtag) construct. Transformed leaves were incubated in a 1% paraformaldehyde solution under vacuum for about 20 min to cross link NSP1 with its target promoters, and the crosslink was quenched by glycine addition and an additional 20 min under vacuum. The induction of both promoters was previously verified with the GUS assay as described above. ChIP was performed using the EpiQuik™ Plant ChIP Kit from Epigentek (Farmingdale, NY, USA), according to the manufacturer's instructions with minor modifications: an additional step of washing in sucrose 30% cushion was added to better purify the nuclei. As a negative control we also co-expressed with or without the 35S::*MtNSP1(HAtag)* construct the p*miR171a*::*GUS* construct that is not induced by NSP1 (data not shown). In order to immune-precipitate NSP1 containing HA tag we used the Rabbit polyclonal Anti-HA tag antibody–ChIP Grade ab9110 from abcam® (Cambridge, UK). We tested the following conditions. Tobacco leaves: p*D27*, p*MAX1* and p*miR171a*, without *NSP1* => IP perfomed with or without Ab against HA; and Tobacco leaves: p*D27*, p*MAX1* and p*miR171a*, with *NSP1* => IP performed with or without Ab against HA. Precipitated DNA was directly analyzed by q-PCR as described above, primers are listed Table S1.

3.8. Mycorrhizal phenotyping and fungus staining

Mycorrhized roots were cleared in 10% w/v KOH for 8 min at 90°C and rinsed with water. Then they were treated over night at 4°C with fluorescein-conjugated wheat germ agglutinin (WGA-FITC, Invitrogen) in 0.0001% PBS, which binds fungal chitin and observed using a stereomicroscope Axio Zoom.V16 Zeiss. Alternatively, roots were stained with Schaeffer black ink as described by Vierheilig *et al.* (1998).

Mycorrhizal phenotyping (number of infection sites, intraradical hyphal propagation and arbuscule density) of plants grown in falcon tubes was performed on ink stained root segments fixed in 30% water/glycerol solution, scanned on microscope slides by using a Nanozoomer 2.0 HT (Hamamatsu, Japan) and analyzed with the NDP view 2.5 software.

3.9. Statistical analyses

For Fig. 3 A four independent biological repeats were performed using 3 replicates from different tobacco leaves. For Figs. 8 and 9, two and three independent biological repeats were performed using 8 to 10 plants for each condition, respectively. For Fig. 7 experiments were repearted three times using 10 plants per conditions. Tests of normality were performed using the Shapiro-Wilk test. According to this test, means were calculated using either the Two-way analysis of variance (ANOVA) or the Kruskal–Wallis test (R software). Significance levels were based on Tukey's post-test (1-way ANOVA). For Fig. 7, significant between myc and no myc for each fungal characteristic were calculated using standard student t test. (Fig.3 **<0.01, Fig. 7 A, B, *<0.05, **<0.01, Figs. 8 A and 9A, a-b <0.05, Figs. 8 B and 9 B, a-c<0.01).

Chapter 2:

MtNSP1 transcripts act as a Target Mimic of the miR171h.

As described in chapter one, an extensive study of GUS expression driven by different promoters with transcriptional and translational constructs was done. Surprisingly, for *NSP1*, we have shown that the two transcriptional and translational constructs were localized in different root tissues. The comprehension of the biological relevance of such differences of localization was quite puzzling at first. In this chapter we try to understand this paradox and we discover an important and new role played by the messenger RNA of *NSP1*.

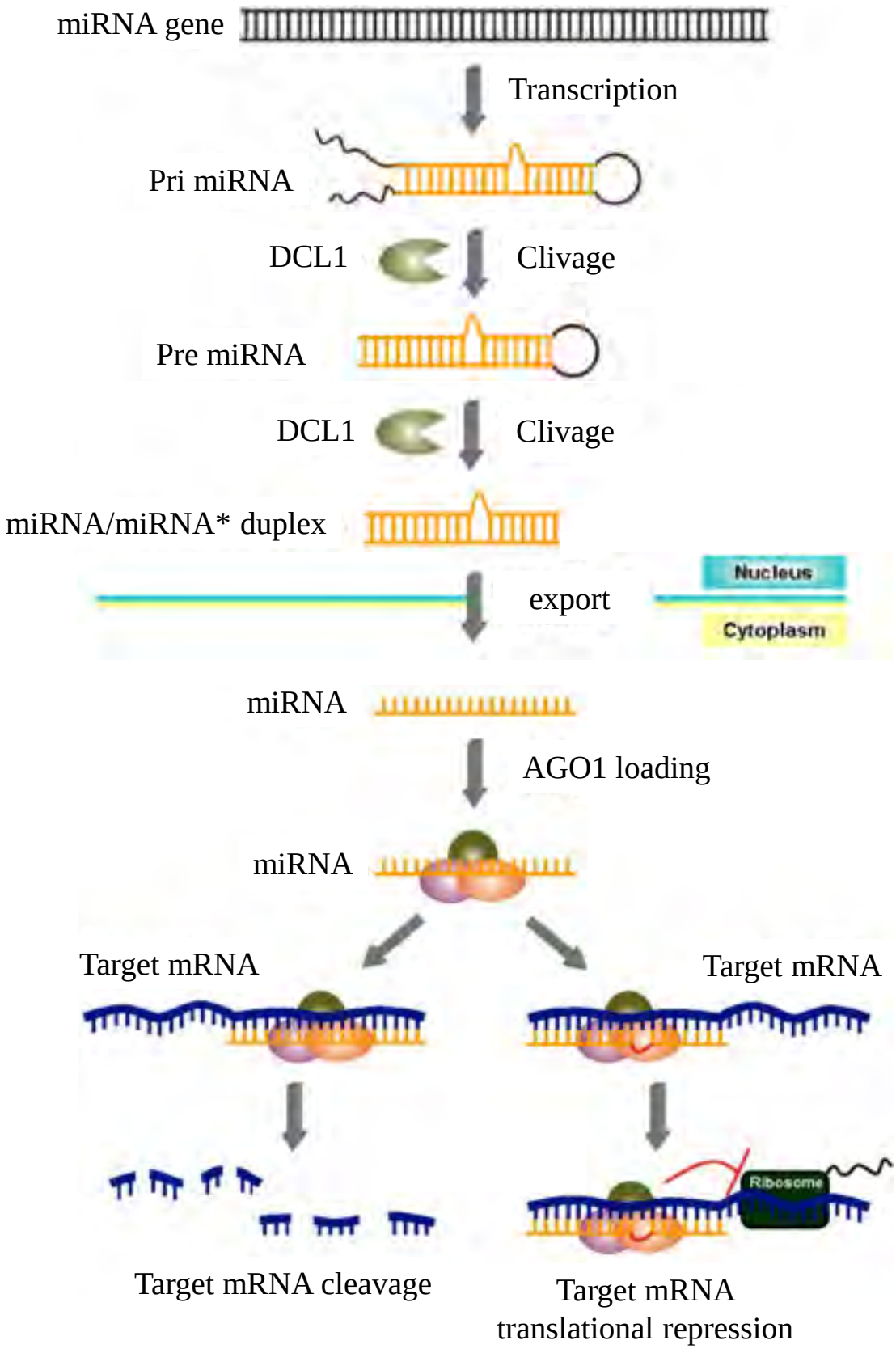


Figure. 1: General mechanism showing the different steps of miRNA production and the two main miRNA action (modified from Liu *et al.*, 2014).

1. Introduction

In the recent years a very active research has progressively unveiled the numerous, previously unexpected, regulatory functions of non-coding RNAs (ncRNAs) (Amor *et al.*, 2009; Li & Zhang, 2016; Gulyaeva & Kushlinskiy, 2016). Different approaches such as high-throughput RNA-sequencing (RNA-seq), degradome studies, *in-silico* predictions or simply serendipity, have highlighted a wide and heterogeneous group of non-coding RNA molecules with different functions. Thousands of novel ncRNAs have been identified in the genome of many organisms, such as humans, animals and plants (Ravasi, 2005; Birney *et al.*, 2007; Matera *et al.*, 2007; Ponting *et al.*, 2009; Guttman *et al.*, 2009). These ncRNAs have been classified in different kinds according to their location, length, and biological functions (Costa, 2005; Amor *et al.*, 2009; Zhu & Wang, 2012; Jin *et al.*, 2013).

Among them, microRNAs (miRNAs) are small RNA molecules with essential roles in organism development and physiology (Mallory & Vaucheret, 2006; Voinnet, 2009; Rubio-Somoza & Weigel, 2011; Zhao *et al.*, 2012). Plant miRNAs are first transcribed as a primary transcript (pri-miRNA) that folds into a hairpin-like RNA secondary structure. This structure is then processed with a specific nuclear enzyme (DCL1) to a pre-miRNA that comprises the miRNA and its complementary miRNA* sequence, and finally to the mature miRNA, a small single-stranded RNA molecule of about 21-nucleotide-long. The mature microRNA is then exported in the cytoplasm and loaded by AGO1 to initiate the inhibition of the expression of specific microRNA target genes. This inhibition occurs either by preventing their translation or by cleaving their mRNAs (Fig. 1) (Rogers & Chen, 2013; Borges & Martienssen, 2015). Most miRNAs have multiple target gene (Chen, 2010). In plants, for an effective gene inhibition, very limited number of mismatches (up to five) or small gaps (made of up to six nucleotides) must occur between the miRNA and target mRNAs, and when considering the miRNA nucleotides 10 and 11 (from 5' to 3') the pairing must be perfect for an effective cleavage (Jones-Rhoades *et al.*, 2006; Axtell & Bowman, 2008; Mallory *et al.*, 2008; Zheng *et al.*, 2012; Brousse *et al.*, 2014) .

In plants, multiple pri-miRNAs produce similar but not always fully identical mature miRNA products (Reviewed in Li & Mao, 2007). Interestingly the expression of these different members of a miRNA family, often annotated with a letter (eg: miR171a, miR171b, etc…), is often under the control of very different promoters. Hence while the different members of a miRNA family generally target the same set of genes, they have singular transcriptional

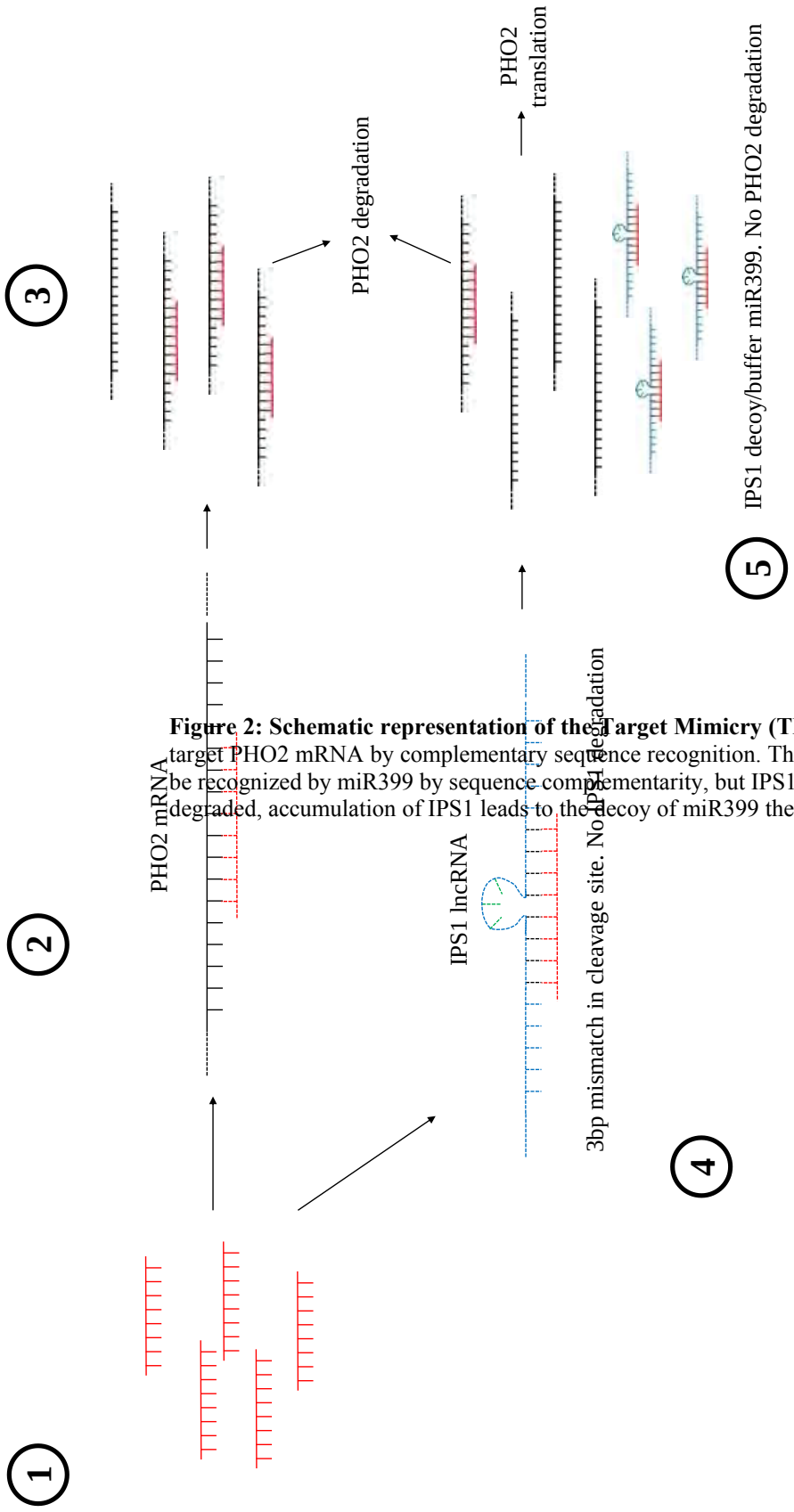


Figure 2: Schematic representation of the Target Mimicry (TM) effect. (1) miR399 is produced in the cell th target PHO2 mRNA by complementary sequence recognition. This targeting leads to the degradation of PHO2 m be recognized by miR399 by sequence complementarity, but IPS1 possess a 3bp mismatch in the cleavage site of degraded, accumulation of IPS1 leads to the decoy of miR399 thereby preventing miR399 degradation of PHO2,

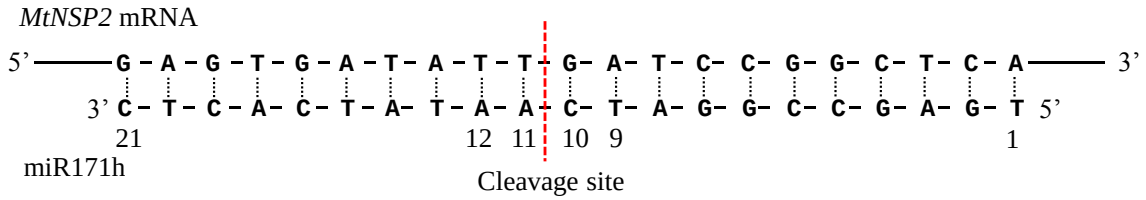
regulation (Maher, 2006; Budak & Akpinar, 2015). It has also been shown that mature miRNAs can move from cells to cells, away from their initial transcription site, which creates gradients of miRNA abundance and miRNA activity across different adjacent cell layers (Marín-González & Suárez-López, 2012; Pyott & Molnar, 2015), leading to a different location between the expression of pri-miRNA, and miRNA activity (Carlsbecker *et al.*, 2010).

Another level of miRNA regulation involves the mimicry phenomenon. Initially described with the miR399 of *A. thaliana*, the mimicry occurs when a long non coding RNA carries a site complementary to the miR399, but containing a 3 nucleotide gap between the 10^{th} and 11^{th} nucleotide of the miRNA cleaving site (Fig. 2). Two natural target mimics (TMs) of miR399 have been identified, At4 and IPS1. These pseudo-targets are recognized by the miRNA but not cleaved, resulting in the sequestration of the miRNAs molecules. This reduces the pool of active miRNAs and therefore decreases the overall miRNA activity toward its genuine target *PHO2* (Franco-Zorilla et al., 2007) (Fig. 2). This natural molecular mechanism also exists in animals (called miR sponges, Ebert *et al.*, 2007), and is thought to participate in the miRNA homeostasis (Seitz, 2009). Artificial TMs are now used to buffer miRNA activity and mimic miRNA mutant phenotype (Todesco *et al.*, 2010; Ivashuta *et al.*, 2011; Yan *et al.*, 2012). From the initial identification of target mimics, bioinformatics analyses of plant genomes have revealed a lot of potential TMs derived from non-coding RNA and even from coding genes (Ivashuta *et al.*, 2011; Meng *et al.*, 2012; Wu *et al.*, 2012; Zhang *et al.*, 2014b). There is indeed no reason to discriminate coding genes from bioinformatics analyses in order to identify new TMs.

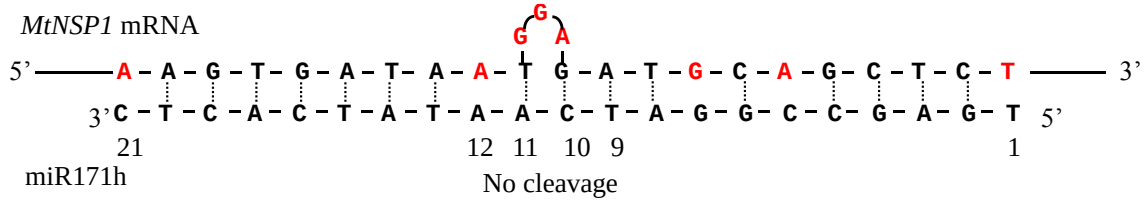
In plants miRNA related gene regulation is involved in a plethora of mechanisms. But more specifically at least 20 miRNA families have been proposed to regulate symbioses that plants establish with soil fungi (called Arbuscular Mycorrhizal Fungi, AMF) and nitrogen fixating bacteria (*rhizobium* sp.) (Simon *et al.*, 2009; Lelandais-Brière *et al.*, 2016).

In 2011, a degradome analysis have highlighted that a transcription factor gene, *NSP2*, was the target of the miRNA 171h (Fig. 3 A) (Devers *et al.*, 2011; Branscheid *et al.*, 2011). NSP2 and its interacting partner NSP1 (NSP: Nodulation Signaling Pathway) belong to the GRAS family and are indispensable for the rhizobial symbiosis. They belong to a signaling cascades leading to the nodule formation (Kalo, 2005; Smit, 2005). They also participate in the regulation of mycorrhizal colonization (Maillet *et al.*, 2011; Delaux *et al.*, 2013). Both miR171h and NSP2 are induced in the root colonized areas and in the arbuscule containing

A **Common miR action:**



B **Potential mimicry action:**



C ***MtNSP1* mRNA mutamiR:**

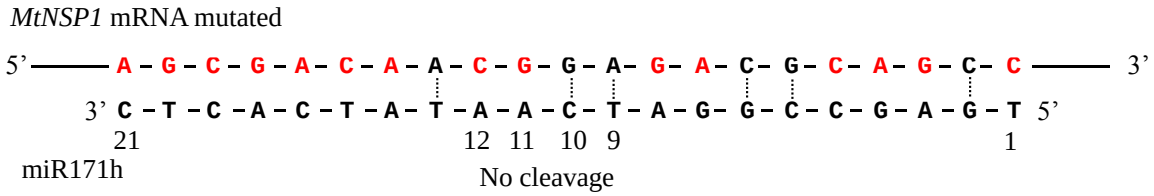


Figure 3: Schematic view of the complementarity between a miRNA and its genuine or its Target Mimic. (A) Recognition by sequence complementarity between the miR171h and the *NSP2* mRNA. The perfect match between the 10th and the 11th position of the miRNA lead to the cleavage of the *NSP2* mRNA. (B) Potential recognition between the miR171h and the *NSP1* mRNA. In red are represented the mismatches, and between the 10th and the 11th position the 3 bases mismatches lead to the inhibition of *NSP1* mRNA cleavage. (C) Representation of the *NSP1* mRNA from which the putative target mimic sequence has been synonymously mutated in order to avoid complementary recognition between the miR171h and *NSP1* mRNA.

cells (Hofferek *et al.*, 2014). The miR171h is also induced by exogenous addition of fungal signaling molecules, the Myc-LCOs, mostly at the root tips and elongation zones. Interestingly, the fungus rarely colonizes these root parts. The overexpression of miR171h results in an inhibition of *NSP2* and in a lower mycorrhization rate. Additionally, in transgenic roots where *NSP2* is mutated to modify the target site of miR171h, a much higher fungal colonization is observed, even in root tips (Lauressergues *et al.*, 2012). Thus it has been proposed that miR171h and NSP2 are important molecular actors of the spatial regulation of AM fungal colonization (Lauressergues *et al.*, 2012).

In this context, we found by *in-silico* analysis that miR171h is also able to recognize a complementary sequence in the exonic mRNA sequence of *NSP1*, but with a 3 base-pair mismatch at the miRNA cleavage site and with only 5 additional mismatches. Here we investigate whether the *NSP1* mRNA plays a target mimicry role and down-regulates the miR171h degradation of *NSP2*.

2. Results

2.1. *MtNSP1* is able to act as a target mimic in tobacco leaves.

As shown in Fig 3 B, *MtNSP1* mRNA presents a bulge of 3 nucleotides corresponding to the cleavage site of *MtmiR171h* between the 10th and the 11th nucleotide (5' to 3') of the microRNA (Fig. 3 B). Theoretically miR171h could then pair with this mRNA sequence but with no further steps of degradation or inhibition of translation. This *NSP1* mRNA sequestration of miR171h would then reduce the free pool of this microRNA and consequently limit the *NSP2* down-regulation.

To check whether *NSP1* mRNA could actually be a coding Target Mimic (cTM) of miR171h, we constitutively expressed in tobacco leaves *NSP2* alone, or *NSP2* in the presence of *pri-miR171h* and/or *NSP1*. In the absence of the *NSP1* construction we first confirmed the negative regulation of *NSP2* by *miR171h* (Fig. 4 A). Then, when *NSP1* was co-expressed with *NSP2* and *miR171h*, we revealed that *NSP2* expression was higher than in the absence of *NSP1*, strongly suggesting that *NSP1* could decoy *miR171h* and thereby protects *NSP2* mRNA from degradation. In order to ensure that our predicted TM sequence of *NSP1* was responsible for its buffering action, we mutated this *NSP1* mimicry sequence by introducing several mismatches preventing the recognition by *miR171h*, but keeping the same amino acid sequence (Fig. 3 C). When we co-expressed this mutated version of *NSP1* (*mNSP1* mutamiR)

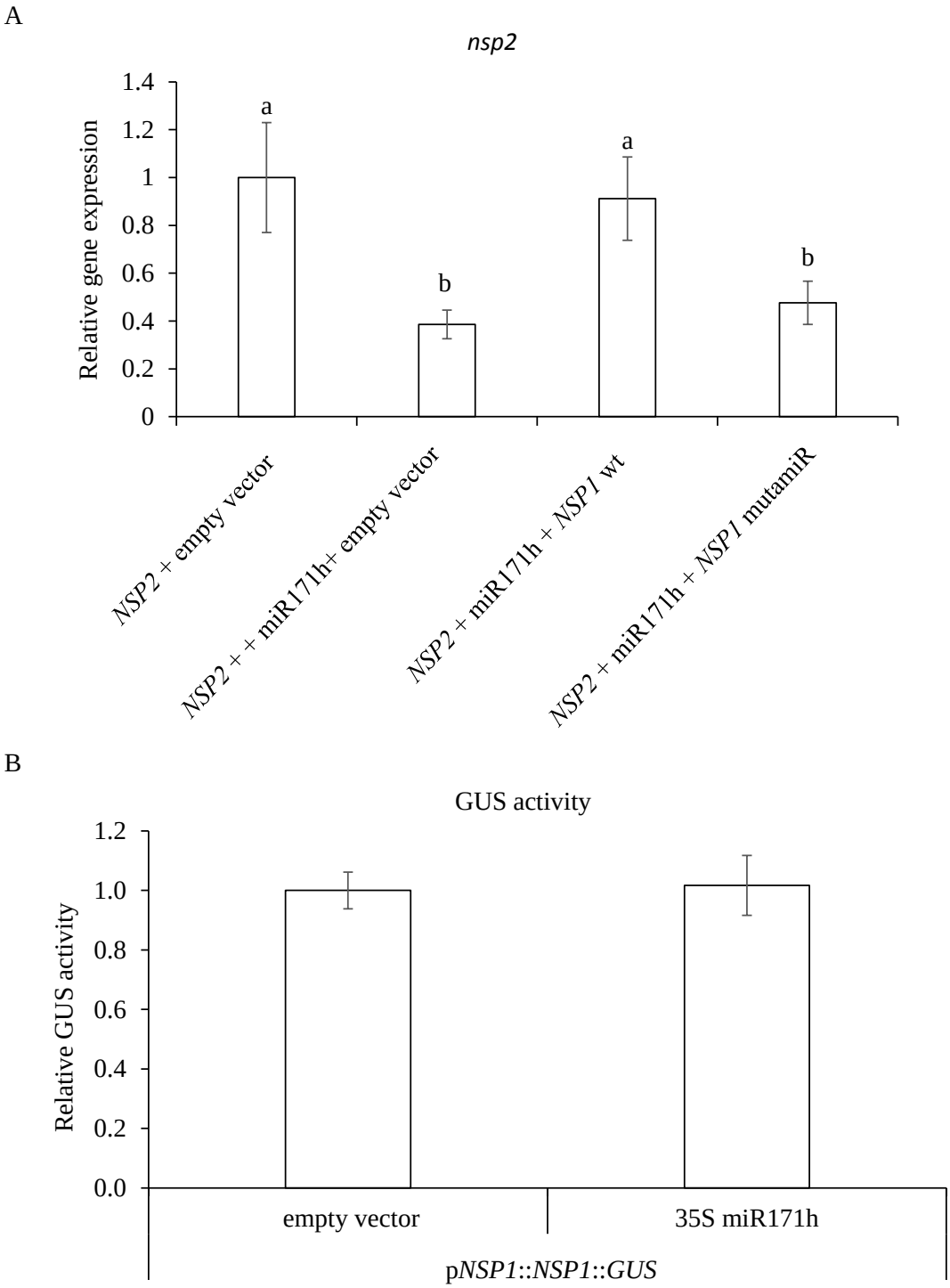


Figure 4: Co-expression of *MtNSP1* is able to limit *Mt*pri-miR171h mediated degradation of *MtNSP2* mRNA in Tobacco leaves. (A) Relative expression measured by qRT-PCR of *MtNSP2* transcript in tobacco leaves, alone or in the presence of *Mtpri-miR171h* and/or *MtNSP1 or MtNSP1*-mutamiR (cf Fig. 3 C). All expressed elements are under the 35S constitutive promoter. (B) Enzymatic GUS activity of tobacco leaves expressing the p*NSP1*::*NSP1*::*GUS* with or without the 35S::*Mtpri-miR171h*. Error bars represent SEM. (A) and (B) n=6, with three biological repeats, (A) significance levels were based on Tukey's post-test (1-way ANOVA), a-b $p<0.05$.

with *NSP2* and *miR171h*, we could suppress the mimicry effect of *NSP1* (Fig. 4 A). We also verified that the mutation introduced in the *NSP1* sequence did not affect its expression (Fig. S1). Finally, to ascertain that the potential interaction between *NSP1* mRNA and *miR171h* did not inhibit the translation of *NSP1*, we expressed in tobacco leaves a construct carrying the promoter of *NSP1* fused to the *NSP1* coding sequence fused to the *GUS* reporter gene sequence. We co-expressed this construct with or without *miR171h* and analyzed the resulting GUS activity. The expression of *miR171h* did not affect GUS expression suggesting that it does not affect *NSP1* translation (Fig. 4 B).

2.2. *Mt-NSP1, Mt-NSP2,* and *Mt-miR171h* expression are colocalized during mycorrhization.

Because *NSP1*, *NSP2* and the *miR171h* have been shown to be involved in the highly dynamic process of mycorrhization, we wanted to verify if the localization of the expression of these genes was consistent with their potential interaction. It has already been published using promoter GUS constructs (and *in-situ* hybridization for the miR171h) that both miR171h and *NSP2* are expressed in the arbuscule-containing cells (Hofferek *et al.*, 2014). When we analyzed the expression pattern of the *NSP1* promoter fused to the GUS sequence, we observed that the mRNA of *NSP1* was expressed in the arbuscule-containing tissues Fig. 5 A). To get a little bit further we analyzed the expression pattern of the construct of *NSP2* carrying the p*NSP2*::*NSP2*::*GUS* sequence. NSP2 proteins seem also to be present in the arbuscule containing-tissues confirming that despite miR171h expression in these tissues the efficient translation of NSP2 was possible (Fig. 5 B). Altogether these data indicate that the interaction between miR171h and the transcripts of NSP1 and NSP2 are physically possible since these molecules are present in the same root tissues.

2.3. *Mt-NSP1* plays a role as a target mimic during mycorrhization.

In the two available and commonly used *nsp1 Medicago* mutants, the non-functional versions of the NSP1 protein correspond to truncated forms of 239 aa in *nsp1-1* and 487 aa in *nsp1-2* instead of 554 aa (Smit, 2005). However, both versions produce transcripts that still possess the predicted mimic sequence of miR171h and could thereby potentially act as a target mimicry and therefore could still protect *NSP2* against its degradation by miR171h.

Therefore, to abolish the potential target mimicry action of *NSP1* mRNA, we created a RNA silencing (siRNA) cassette against *Mt-NSP1* mRNA. We then compared the

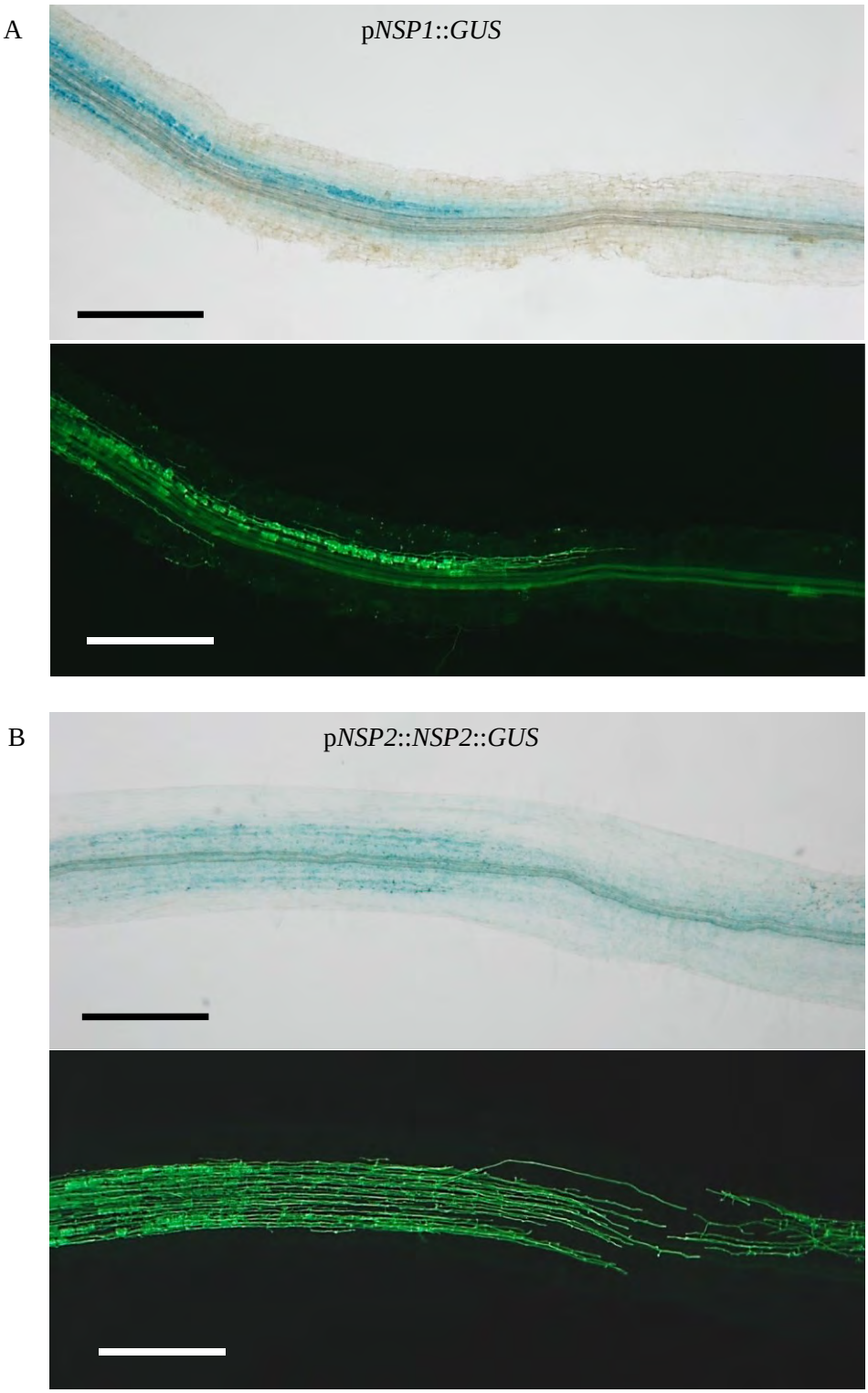


Figure 5: GUS expression pattern of the NSP1 transcriptional fusion and the NSP2 translational fusion in *M. truncatula* chimeric plants during mycorrhization. (A) The expression of the p*NSP1*::*GUS* construct appears to be localized in the fungal containing structures and more precisely is strongly induced in the arbuscule containing cells. (B) The expression pattern of pNSP2::NSP2::GUS constrcutc appears to be localized in the fungal containing structures. Upper picture correspond to the bright field image, and bottom pictures are the respective image under fluorescent light showing the fungus stained with WGA-FITC. Scales =200µm.

mycorrhizal phenotypes of the wild-type plants, the *nsp1* mutant that still potentially possesses the mimicry sequence and the *nsp1* mutant expressing the *NSP1* siRNA cassette. In our three repeats, *nsp1* mutant silenced for *NSP1* displayed a reduced mycorrhization rate compared to either the *nsp1* mutant or the WT transformed with an empty vector. This accentuated defect in mycorrhization when both the NSP1 protein is non-functional and the mRNA of *NSP1* is not expressed, points out the potential role NSP1 transcripts as target mimicry of the miR171h.

2.4. Prediction of potential new coding target mimics (cTMs).

Because we could show that a coding sequence is able to act as a Target Mimic (TM) we performed a bioinformatic analysis to investigate, in the plant model *Arabidospis thaliana* and *Medicago truncatula*, the possibility that other coding sequences could potentially be target mimics. Our screen was set, using the mRNA library from miRBase and the coding genome of both species. We first search for coding mRNA sequences having a 3 nucleotides gap in the critical position 10-11 of a microRNA. We allowed the presence of up to five mismatches in the target mRNA sequences (Axtell & Bowman, 2008; Mallory *et al.*, 2008; Brousse *et al.*, 2014), but we removed those presenting the five mismatches on only one side of the cleaving site (5'or 3'). By performing this analysis on *both* genomes we could identify thousands of potential cTMs for the whole set of miRNAs, and sometimes several hundred of potential cTMs per miRNA (Table 1). We also performed the same analysis on the non-coding RNA of *A. thaliana* and found to a less extent a few TMs for most of the conserved miRNA family.

3. Discussion:

We provided for the first time that a coding mRNA can act as a Target Mimic of a miRNA and prevent degradation of the natural mRNA target. In the example described here two genes encoding transcription factors NSP1 and NSP2, known to positively regulate important symbiotic processes (nodulation and mycorrhization), are interacting with the same microRNA (*Mt-miR171h*), one (*Mt-NSP1*) as a mimic target and the other (*Mt-NSP2*) as a genuine target. When the three genes are transcribed in the same cells, two mechanisms coexist: the down-regulation of *NSP2* by miR171h and the inhibition of this down-regulation by the target mimic *NSP1*. If we consider parameters such as the relative transcriptional activity and transcript turnover of the three genes, the possible migration from cell to cell of

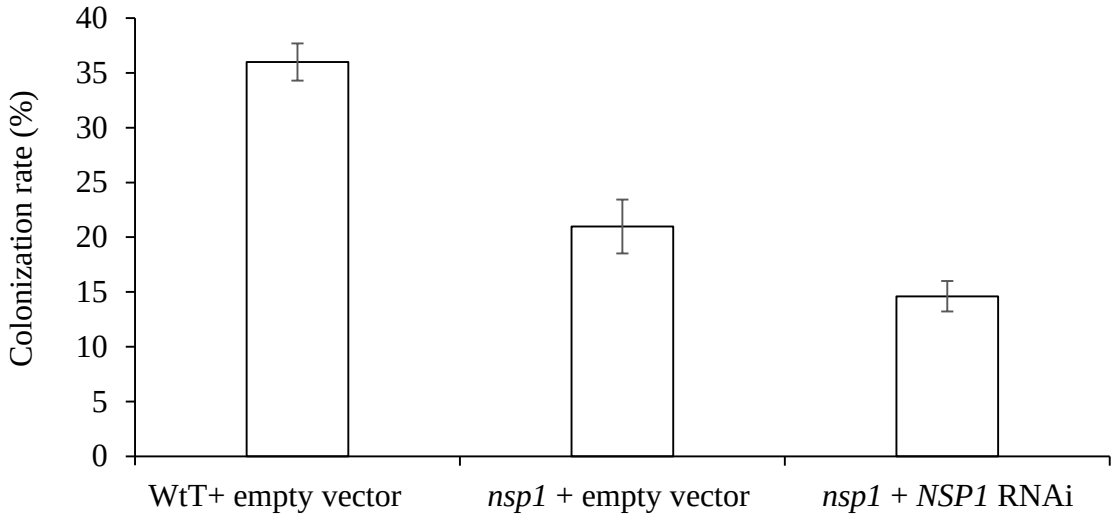


Figure 6: Mycorrhizal phenotype of WT and nsp1 mutant expressing or not the RNAi *MtNSP1* cassette in *M. truncatula* chimeric plant 12 weeks after inoculation with *R. irregularis*. Mycorrhizal rate measured by the grid-intersect method (Giovannetti and Mosse, 1980). Error bars represent SEM.

	# of cTMs		# of ncTMs
miRNA family	***A. thaliana***	***M. truncatula***	***A. thaliana***
miR156	92	972	2
miR159	488	293	5
miR160	141	165	1
miR162	99	140	0
miR164	516	182	5
miR166	138	401	0
miR167	361	282	3
miR168	247	263	4
miR169	363	559	0
miR171	267	942	0
miR172	1279	1522	9
miR319	134	893	5
miR390	164	38	2
miR393	660	286	6
miR395	93	567	1
miR396	1051	1456	7
miR397	249	234	3
miR398	265	86	6
miR399	283	789	7

Table 1: Summary table representing the number of target mimics found in the *in-silico* prediction for both the *A. thaliana* and *M. truncatula* coding genomes and the *A. thaliana* non-coding genome. The 19 conserved miRNA families are represented, and for each genome an arbitrary color scaling has been add showing the abundance of TM found per family (from low to high number of TM, green to red).

the mature miR171h that can modify its concentration over time and space, we figure how dynamic and subtle the spatio-temporal regulation of NSP2 must be. This complex regulation must also play a role in root development, since miR171h has been found to be expressed in the root meristematic and elongation zones like both *NSP1* and *NSP2* (Untergasser *et al.*, 2012) (Chap1. Fig. 1). As miR171h is expressed at low levels along the root and *pNSP1*::*GUS* expression seems to be localized in the central cylinder, where *NSP2* is expressed as well (Fig. S2). We could speculate that an overlapping expression in some regions along the roots is necessary for the subtle regulation of miR171h activity on *NSP2*.

Moreover, miR171h has been predicted to target at least three other genes, such as a *NSP2*-like (Medtr5g058860) and tow genes encoding pentatricopeptide-repeat proteins, Mtr.25350.1.S1_at and Mtr.11537.1.S1_at. Their regulation could also depend on the presence of cTMs. From our *in-silico* analysis, we predicted in *Arabidospis* 267 potential cTMs of the miR171 family (from which 171h is absent) and 90 miR171h potential cTMs in *Medicago*.

These results highlight the potential for numerous cross regulations between miRNAs and a consortium of targets and pseudotargets. This high number of potential cTMs for each miRNA raises the intriguing question of the biological and functional relevance of such a system. We hypothesize that during plant evolution miRNAs and natural coding mimics have been concomitantly developed to restrict miRNA activities, where it was biologically relevant. It has been shown that certain miRNAs can migrate between different cell layers (like miR166 and miR390) but also through the vascular system like miR395 and miR399 (Reviewed in Marín-González & Suárez-López, 2012). Given this natural spreading plants may have developed strategies avoid inappropriate miRNA activities in neighboring cells. We speculate that the high occurrence of cTMs might sustain this strong requirement for a plant to restrict miRNA activities just where they are necessary. The cTMs would mainly be efficient to trap escaping, less concentrated, miRNAs in the neighboring cells. The activity of miRNAs to be restricted in the proper cells would occur where their concentration is the highest, i.e. close to where the pri-miRNAs have been transcribed (Fig. 7).

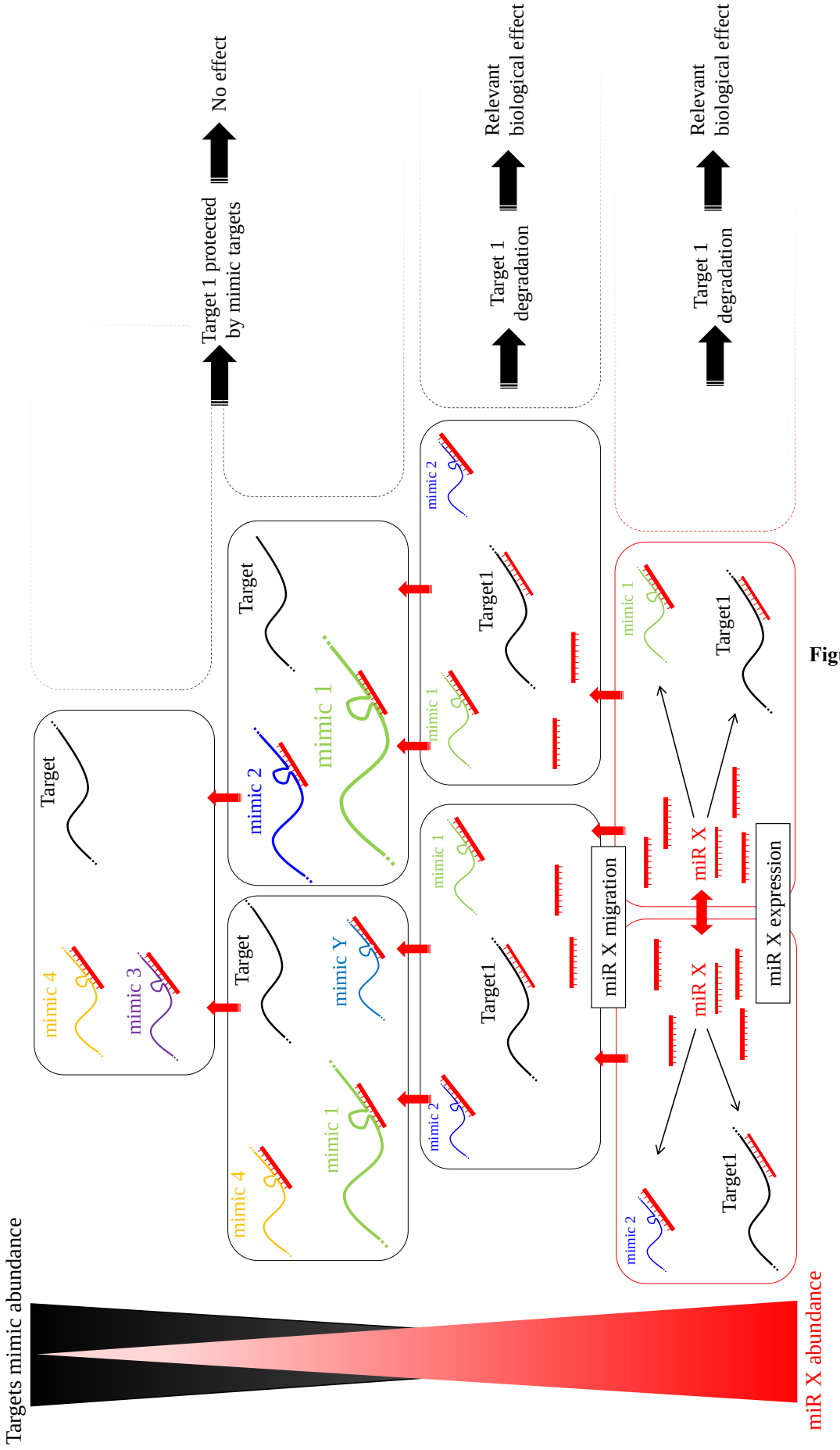

Targets mimic abundance
miR X abundance
Target
mimic 4
mimic 3
Target
mimic 2
mimic 1
Target
mimic 4
mimic 1
mimic Y
Target1
mimic 2
mimic 1
Target1
mimic 1
mimic 2
miR X migration
miR X
miR X
miR X expression
mimic 1
Target1
mimic 2
Target1
No effect
Target 1 protected by mimic targets
Relevant biological effect
Target 1 degradation
Relevant biological effect
Target 1 degradation

Figure 7

1. Materials and methods:

1.1. *In-silico* TMs prediction:

Arabidopsis thaliana and *Medicago truncatula* miRA sequences were collected from miRbase (version 21). Redundant miRNAs from the same family were removed to keep one canonical sequence, but miRNAs of the same family that have at least one base different were kept. Screening was done using all cdna sequences downloaded from the Arabidospis Information Resource (TAIR 10) and the Medicago Hapmap Mt4.0v1. The analysis of the non-coding genome was also done on the Arabidospis Information Resource (TAIR 10) using, non-coding RNA, small nucleolar RNA (snoRNA) and small nuclear RNA (snRNA). Target Mimicry (TM) of the miRNAs were predicted using local scripts with the following rules: (1) bulges were only permitted at the 10th to 11th positions of miRNA sequences; (2) the bulge in TMs should be composed of only three nucleotides; (3) perfect nucleotide pairing was required at the 9th to 12th positions of miRNA sequences; and (4) except for the central bulge, the total mismatches and G/U pairs within TM and miRNA pairing regions should be no more than five. Splice variant of genes were not removed.

1.2. Biological material:

M. truncatula Gaertn 'Jemalong' genotype A17 and *nsp1-1* (Smit, 2005) seeds were used in this study. Seed coats were first scarified by incubation in concentrated (98%) H_2SO_4 prior to be surface-sterilized using NaClO. Seeds were then washed and germinated on agar plates in the dark for 5 days at 4°C. For GUS expression analysis chimeric plants were cultivated in 250 mL pots (one chimeric plant per pot) filled with Oil-Dri US-special substrate

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Figure 7: Schematic model of coding Target Mimic (cTMs) having a proper role as controlling miRNA spreading and restricting miRNA activity in the relevant cells. Bottom, the miR X is expressed in a certain cell type (red) and bind to its genuine target (Target 1) for degradation or inhibition of translation. In the same cells, cTMs are also expressed at low level and due to the high miR X expression they only lightly affect miR X activity. Upper cells, miR X is able to migrate from cell to cell and according to the ratio miR X/ cTMs the miRNA is still able to bind its target. Up, after a certain distance from the expressing cells the abundance of cTM is higher than of the miR X leading to inhibition of the miRNA activity.

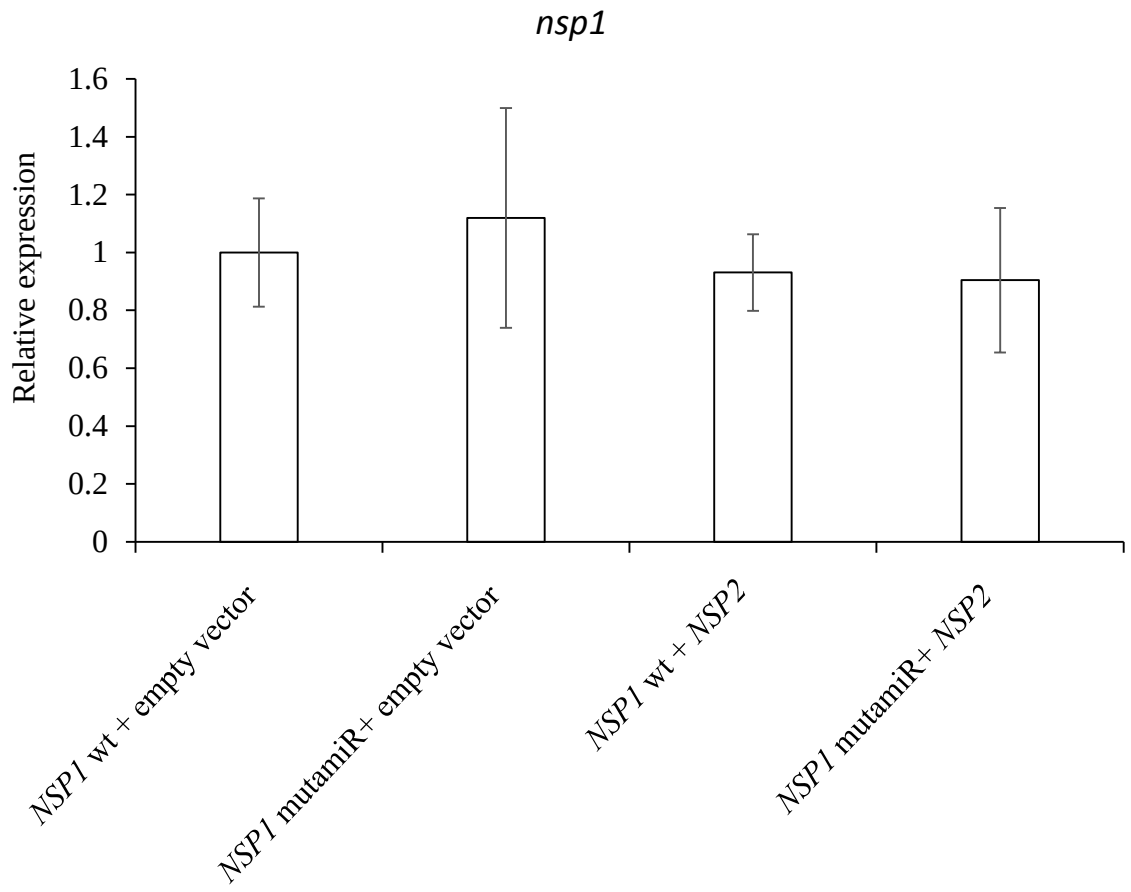

Figure S1: *MtNSP1* and *MtNSP1* mutamiR expression in agroinfiltrated Tobacco leaves. qRT-PCR assay measuring the expression of *MtNSP1* and *MtNSP1* mutamiR coexpressed with and empty vector or with *MtNSP2*. All genes are under the 35S promoter. Error bars represent SEM. No statistically significant differences have been found.

(Damolin, www.damolin.fr) for 5 weeks in a growth chamber (16/8 h day/night, 24℃/22°C, 120-150 µmol $m^{-2}s^{-1}$), and watered every 2 days with modified Long Ashton medium containing a low concentration of phosphate (7.5 µM) (Balzergue *et al.*, 2011). Plants were inoculated with *Rhizophagus irregularis* DAOM 197198 sterile spores (2000 spores per liter of substrate) purchased from Agronutrition (Carbone, France).

Nicotiana benthamiana plants were grown in standard compost with perlite (4:1), in a growth chamber (16/8 h day/night, 24°C/22°C, 200-220 µmol $m^{-2}s^{-1}$) for 5 to 6 weeks (plant display typically at least 5-6 leaves).

1.3. Plasmid construction:

A modified pCAMBIA2200 binary vector was used with the Golden Gate strategy for cloning (Engler *et al.*, 2008). The DNA fragments of interest were flanked by BsaI restriction sites during the PCR amplification step using Pfu polymerase on *M. truncatula* DNA (Promega, www.promega.com). The primers are shown Table S1). One-step digestion–ligation reactions were carried out with 100 ng modified pCAMBIA, 100 ng of each PCR fragment, 1 µl 10× ligase buffer (Promega), 2.5 U T4 DNA ligase (Promega), 2.5 U BsaI (NEB), in a final volume of 10 µl and incubated at 37 °C for 30 min and 16 °C for 30 min and repeated once. A final incubation step at 50 °C for 20 min was used to cleave any remaining undigested cloning vector. To build the *NSP1* mutamir construct, *NSP1* sequence before and after the mimicry region were firstly amplified separately by PCR using primers containing the mutamir sequence. Then an overlap PCR was performed in order to obtain the full sequence of *NSP1* containing the mutamir sequence and BSaI restriction sites for Golden Gate insertion. All genes expressed in tobacco leaves were under the control of the Cauliflower mosaic virus 35S. Promoter amplified were p*NSP1* (3183 bp), p*NSP2* (2890 bp) and *NSP1* post CDS section (3180 bp). All the primers used are listed Table S1. Genes used were miR171h (MIMAT0021269 on miRBase), *Mt-NSP1* (Medtr8g020840), *Mt-NSP2* (Medtr3g072710).

1.4. Tobacco agroinfiltration and GUS assays:

Nicotiana tabacum leaves were agroinfiltrated following the protocols (Yang *et al.*, 2000). Forty hours after infiltration, 3 disc of each agroinfiltrated leaves were harvested and frost in liquid nitrogen prior to GUS assays or RNA extraction.

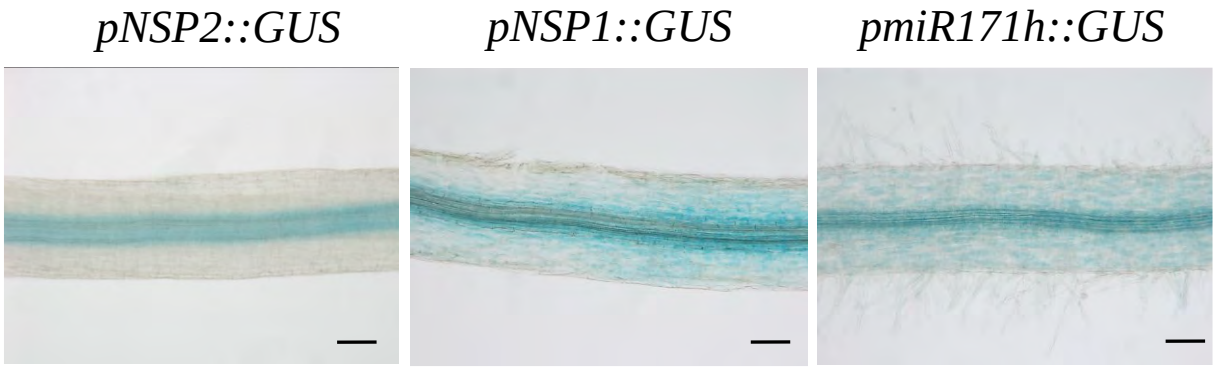

Figure S2: GUS expression pattern of the *NSP1*, *NSP2* and *miR171h* transcriptional fusion. In *M. truncatula* chimeric root, in Asymbiotic conditions, all three genes are expressed along the root. MiR171h is expressed at low levels along the root and p*NSP1*::*GUS* expression seems to be more localized in the central cylinder, where NSP2 is expressed as well. Scales=100µm.

genes	Sequence 5'-3'
Golden Gate cloning	
GoldGate MtNSP1 prom+gene Fw BsaI	AAAGGTCTCAAAATATGCCTTTATCATTTTTGGGG
GoldGate MtNSP1 prom+gene Rv BsaI	AAAGGTCTCACCATTTCTGGTTGTTTATCCAGTTTCC
GoldGate MtNSP1 3'UTR Fw BsaI	AAAGGTCTCCTTCGTTCGAGCTCAACATTGACAGCA
GoldGate MtNSP1 3'UTR Rv BsaI	AAAGGTCTCCCGTACCTCCTCCTTATACTTTCTTG
GoldGate MtNSP2 gene Fw BsaI	AAAGGTCTCCAATGGATTTGATGGACATGGATG
GoldGate MtNSP2 gene Rv BsaI	AAAGGTCTCTCCATTAAATCAGAATCTGAAGAAGAAC
GoldGate MtNSP1 RNAi sens Fw BsaI	AAAGGTCTCCTAGCATGACTATGGAACCAAATCCAA
GoldGate MtNSP1 RNAi sens Rv BsaI	AAAGGTCTCCGCGTTGCTATTTGTGGCATTAGAAG
GoldGate MtNSP1 RNAi antisens Fw BsaI	AAAGGTCTCCATTAACGATAAACACCGTAATCTTCT
GoldGate MtNSP1 RNAi antisens Rv BsaI	AAAGGTCTCCCGTATACTGATACCTTGGTTTAGGTT
GoldGate MtNSP1 Fw BsaI	aaaGGTCTCcTAGCATGACTATGGAACCAAATCCAAC
GoldGate MtNSP1 mutamiR Rv BsaI	CTGTCCCTTCAagGgctgcGtctccGttGtcGcttTCATTCATCGG
GoldGate MtNSP1 mutamiR Fw BsaI	CCGATGAATGAaagCgaCaaCggagaCgcagcCctTGAAGGGACAG
GoldGate MtNSP1 tag His Rv BsaI	aaaGGTCTCgCGTAtcacacgtggtggtggtggtggtgCTCTGGTTGTTTATCCAGTTTC
GoldGate Prom miR171h Fw BsaI	AAAGGTCTCAAAATTGATTTGTTGTTTGATCTATTTC
GoldGate Prom miR171h Rv BsaI	AAAGGTCTCCACACCGCCATGTATACTTTTGCAGC
qTR-PCR	
NbEF1 qpcr Fw	CAGCTTCTGCCACAGCTACA
NbEF1 qpcr Rv	GGTTGGTGAGTGGAGGAAAA
MtNSP1 qpcr Fw	ATTCAACCAGTTCGGCATTC
MtNSP1 qpcr Rv	CTGCAAACCCTGCTTCTTTC
MtNSP2 qpcr Fw	GTCCTCGAACAGCTCAGTCC
MtNSP2 qpcr Rv	GCGTTTTTATTGCCGTTGTT
MtmiR171h qpcr Fw	CAATTTCAGACGAGCCGAAT
MtmiR171h qpcr Rv	GAGCAGAAACAACACCACTCC

Table S1: Primers used in this article for both the Golden-Gate cloning and the qRT-PCR.

GUS assays were performed by extracting the total proteins from 100 mg of transformed leaves after grinding in liquid nitrogen, with 100 µl of GUS buffer (100 mM Phosphate buffer pH7, 0.1% TritonX-100, 10 mM β-mercaptoethanol) (Wagner *et al.,* 2015). Glucuronidase activity was measured by fluorometric assay with 25 µl of protein extracts and 1 mM MUG (4-methylumbelliferyl glucuronide, Sigma) in a total reaction volume of 200 µl. Fluorescence was measured every 5 min during 120 min on a TriStar LB 941 Multimode Microplate Reader (Berthold Technologies) at 37°C with 360 nm excitation and 460 nm emission. The fluorimeter was calibrated with freshly prepared MU4 (4-methylumbelliferone sodium salt, Sigma-Aldrich) standards in the same GUS buffer. Normalization was done by measuring the total protein concentration by the Bradford method on 96 well plates. Two hundred microliters of Bradford reagent (Bio-Rad Laboratories) were added to 5 µl of samples. After incubation (15 min, 25°C), absorbance was measured at 565 nm. Standard curve was done with 1–20 µg of BSA (Sigma-Aldrich). Glucuronidase activity was calculated from the linear part of the reaction (between 20 and 100 min) and expressed as nkatal/mg of total proteins.

1.5. Hairy root transformation:

Chimeric plant were produced as described in Boisson-Dernier et al. (2001) two days before transformation, *Agrobacterium rhizogenes* ARqua1 (Quandt, 1993) cultures containing the constructs of interest were grown on solid LB supplemented with the antibiotic of selection kanamycin (25µg/ml) at 28°C.

In a Petri dish with sterile water, approximately 1 cm of root tip of each five-day-old seedling was removed. Wounded root tips were then dipped in the bacterial layer, and immediately transferred on 12 cm square plates filled with Farhaeus medium gelled with Bacto™ Agar, Becton, Dickinsson and Company, Sparks, MD, USA (7%) and supplemented with the selective antibiotic kanamycin (25 µg/ml). The plates were incubated in a 24°C, 16 h/8 h day/night, 60 µmol $m^{-2}s^{-1}$growth chamber for 3 weeks. Seedlings were then screened with a stereomicroscope Axio Zoom.V16 (Zeiss, Oberkochen, Germany) under RFP Plus filter set (Uex=546/12; Uem=607/80), to discard all the out-growing, non- DsRED expressing, wild-type roots. Before fungal inoculation, the selected chimeric plants were acclimated in Oil-dri US special substrate for one week under saturated hygrometry.

1.6. Gene expression analyses*:*

For quantitative RT-PCR analyses, total RNA was extracted using a Plant RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated by DNase I

(Promega) to remove genomic DNA contamination. Reverse transcription was performed using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega) on 1 µg of total plant RNA. For each experiment, six to twelve independent plants were analyzed. Quantitative PCR amplifications were conducted on a Roche LightCycler 480 System (Roche Diagnostics) under the following conditions: 95°C for 5 min, then 45 cycles of 95°C for 15 sec and 60°C for 1 min. The measured transcripts were normalized by using the tobacco *EF1α* gene. The primers used in this study are listed Table S1.

For histochemical GUS analysis, root tissues of the different GUS expressing chimeric plants were first fixed in 1% formaldehyde in 100 mM sodium phosphate buffer, pH 7.2, under vacuum for 15 min and then soaked in GUS staining solution (100 mM sodium phosphate

buffer, pH 7.2, 10 mM EDTA, 0.1 % Triton X-100, 0.3 mg ml^{-1} X-Gluc) under vacuum for 15 min. Root tissues were then incubated from 30 min to 24 h in GUS staining solution at 37 °C.

Any subsequent fungal staining using WGA-FITC was performed on GUS stained roots, cleared with KOH and stained with WGA-FITC as described below for mycorrhizal phenotyping.

1.7. Mycorrhizal phenotyping and fungus staining

Mycorrhized roots were cleared in 10% w/v KOH for 8 min at 90°C and rinsed with water. Then they were treated over night at 4°C with fluorescein-conjugated wheat germ agglutinin lectin (WGA-FITC, Invitrogen) in 0.0001% in PBS, which binds fungal chitin. Alternatively, roots were stained with Schaeffer black ink as described by (Vierheilig *et al.*, 1998). Observation were made using a stereomicroscope Axio Zoom.V16 Zeiss.

1.8. Statistical analyses

For Fig. 4 and Fig. S1 three independent biological repeats were performed using 6 replicates from different tobacco leaves (three tobacco seedlings with two leaves each). For the Fig. 6, three independent biological repeats were performed using 8 plants for each condition. Test of normality was performed using the Shapiro-Wilk test. According to this test comparisons of means were calculated using either the Two-way analysis of variance (ANOVA) or with the Kruskal–Wallis test, performed in R software. Significance levels were based on Tukey's post-test (1-way ANOVA). (Fig.4 and 6, $p<0.01$, Fig. 6 a-c $p<0.05$).

Chapter 3:
Sl-IAA27 regulates strigolactone biosynthesis and mycorrhization

For this third chapter we had the chance to work with a neighbor laboratory, the laboratory of Genomics and Biotechnology of Fruits (GBF). In order to identify new auxin signaling actors involved in the development and the ripening of tomato fruits, GBF started a systematic screening of all the AUX/IAA proteins of Tomato. In collaboration with our team, one of them, *Sl-IAA27* (Bassa *et al.*, 2012), was shown to be induced in mycorrhizal roots (Bassa *et al.*, 2013). In the present chapter we further analyze the role of *Sl-IAA27* during mycorrhization of tomato and reveal in the importance of this protein in the control of SL production via *NSP1*, *D27* and *MAX1*.

Sl-IAA27 regulates strigolactone biosynthesis and mycorrhization

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Manuscript submitted to New phytologist, and reviewed.

Summary

- Root colonization by Arbuscular Mycorrhizal (AM) fungi is a complex and finely tuned process. Previous studies have shown that among other plant hormones auxin plays a role in this process but the specific involvement of Aux/IAAs, the key regulators of auxin responses is still unknown.
- The expression and role of tomato *Sl-IAA27* during AM symbiosis was addressed using *pSl-IAA27::GUS* and *Sl-IAA27*-RNAi tomato lines, respectively.
- The data show that *Sl-IAA27* expression is up-regulated by the AM fungus and silencing of *Sl-IAA27* has a negative impact on AM colonization. *Sl-IAA27*-silencing resulted in down-regulation of three genes involved in strigolactone synthesis *NSP1, D27* and

MAX1, and treatment of *Sl-IAA27*-silenced plants with the strigolactone analog GR24 complemented their mycorrhizal defect phenotype.

- Overall, the study identified an Aux/IAA gene as a new component of the signaling pathway controlling AM fungal colonization in tomato. This gene is proposed to control strigolactone biosynthesis *via* the regulation of *NSP1*.

1. Introduction

The Arbuscular Mycorrhiza (AM), a symbiosis between soil fungi of the *Glomeromycota* phylum and nearly 80% of terrestrial plant species, is characterized by a two-way trade in which the fungus provides mineral nutrients to the plant in exchange for carbohydrates. The initiation of this symbiosis is known to require a molecular communication between the two partners. The plant secretes several signal molecules in its root exudates including strigolactones (SL), a class of plant hormones playing an important role in the rhizosphere for the establishment of AM symbiosis (Gomez-Roldan *et al.*, 2008). SL stimulate AM fungal metabolism and hyphal proliferation (Akiyama *et al.*, 2005; Besserer *et al.*, 2006; 2008) and from its side, the AM fungus produces trace amount of chitinic signals (Maillet *et al.*, 2011; Genre *et al.*, 2012; 2013). Upon this successful mutual recognition, the fungus penetrates the roots through the epidermis, grows between root cells and forms highly branched structures called arbuscules inside cortical root cells, where most nutrient exchanges occur between the two partners.

The control of the **mycorrhizal** symbiosis is a finely tuned process at multiple levels. An increasing number of reports point to the important role of several plant hormones, besides that of SL, in the regulation of early recognition/colonization step up to the final arbuscular formation (reviewed in Hause *et al.*, 2007; de Los Santos *et al.*, 2011; Foo *et al.*, 2013; Gutjahr, 2014). For instance, auxin is involved in both the general development of the fungus *in planta* and the formation of arbuscules, whereas SL are involved in pre-symbiotic growth of the fungus but not in arbuscule differentiation.

With regard to auxin, several studies have shown an increase in auxin content in AM roots and a stimulation of fungal growth and mycorrhization by exogenous auxin treatment (reviewed in Gutjahr, 2014). This was recently confirmed by the observation that the synthetic auxin-responsive gene DR5-GUS promoter undergoes a net activation in mycorrhized roots and

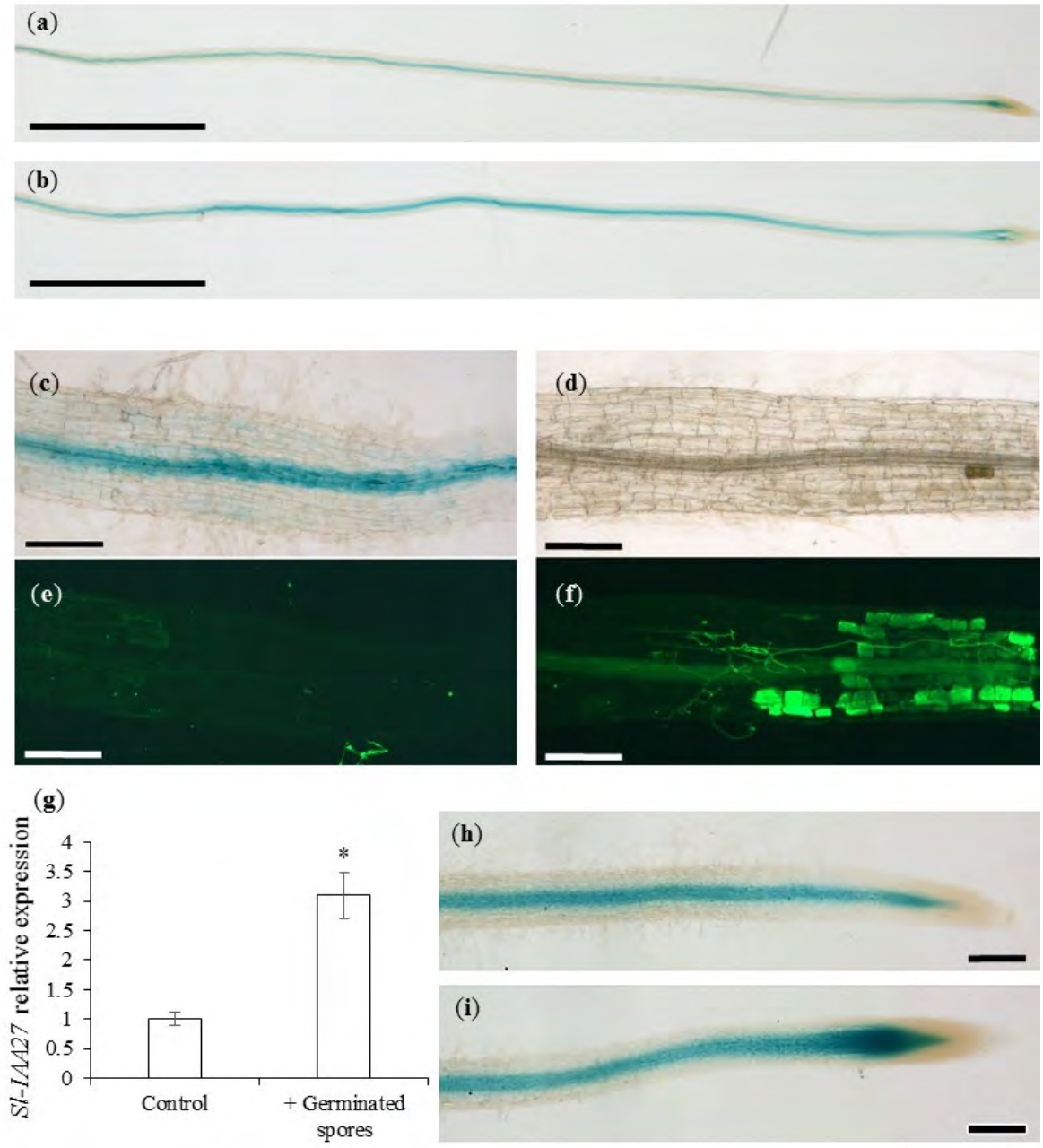

Figure 1: Localization of *Sl-IAA27* expression in roots of *pSl-IAA27*::*GUS* plants inoculated or not with *R. irregularis*. (a, b) GUS expression mainly found in the inner cortex and the central cylinder of young roots, (a) in a root of a non-inoculated plant, (b) in a non-colonized root of an inoculated plant. (c, d) GUS expression in non-colonized (c) and colonized (d) root sections of an inoculated plant. (e, f) Corresponding fluorescent images confirming the absence (e) and the presence (f) of the fungus stained with WGA-FITC. (g) Quantification by qRT-PCR of *Sl-IAA27* gene expression in roots of plants cultivated in vitro in the presence or absence (control) of germinating spores separated with a cellophane membrane. Error bars represent SEM. Stars indicate a significant difference when compared to control according to the Kruskal–Wallis test: $n = 5$ $p < 0.05$. (h, i) GUS expression in a control root (h) and in a root cultivated in the presence of germinating spores (i) separated by a cellophane membrane. The GUS pictures shown in this figure were obtained with line 10/10/1. Scales (a, b) = 2 cm, (c, d, e, f, h, i) = 200 µm.

more precisely in arbuscule-containing cells (Etemadi *et al.*, 2014). Further supporting the role of auxin in the AM symbiosis, the mycorrhization rate was strongly decreased, although showing normal fungal structures and arbuscules, in the pea *bushy* mutant that produces 3 times less auxin in its roots, and also in the auxin resistant tomato mutant *diageotropica* as well as in the auxin hyper-transporting tomato mutant *polycotyledon* (Hanlon and Coenen, 2011; Foo, 2013). Interestingly the low mycorrhization rate of *bushy* was attributed to a decreased SL biosynthesis suggesting a possible cross-talk between auxin and SL in the regulation of AM (Foo, 2013).

Auxin perception and/or signaling appear(s) to be critical for arbuscule development since the inhibition of auxin receptors by the overexpression of the microRNA393 leads to a defect in arbuscule formation in addition to a reduced mycorrhization (Etemadi *et al.*, 2014). Upon auxin recognition the auxin receptors TIR/AFBs become associated with the SKP1-Cullin-F-box (SCF) complex leading to a rapid proteasome-mediated degradation of Aux/IAAs (Dharmasiri *et al.*, 2005a and 2005b; Kepinski and Leyser, 2005; Leyser, 2006; Tan *et al.*, 2007; Chapman and Estelle, 2009), a release of ARFs (Auxin Response Factor) that can then activate the transcription of auxin-regulated genes through binding to auxin-responsive elements present in their promoter region (Hagen *et al.*, 1991, Ulmasov *et al.*, 1997, Hagen and Guilfoyle, 2002). Therefore, one can hypothesize that Aux/IAAs and/or ARFs play a role in the regulation of mycorrhization.

In tomato, 25 *Aux/IAA* genes were identified (Wang *et al.*, 2005; 2009; Herrera-Medina *et al.*, 2007; Chaabouni *et al.*, 2009a and 2009b; Audran-Delalande *et al.*, 2012; Bassa *et al.*, 2012; Deng *et al.*, 2012a and 2012b; Su *et al.*, 2014). Among these, *Sl-IAA27* was shown to display an intriguing expression pattern: a down-regulation upon exogenous auxin treatment and an up-regulation during mycorrhization (Bassa *et al.*, 2012; Bassa *et al.*, 2013).

To gain further insight into the role of auxin, and more specifically that of Aux/IAAs, in the mycorrhization process, we analysed the expression pattern of *Sl-IAA27* in mycorrhized and non mycorrhized roots, the mycorrhizal phenotype of *Sl-IAA27*-silenced plants, and we compared the ability to produce SL of WT and *Sl-IAA27*-silenced plants. The data suggest that *Sl-IAA27* positively regulates mycorrhization via the induction of *NSP1* transcription and SL biosynthesis.

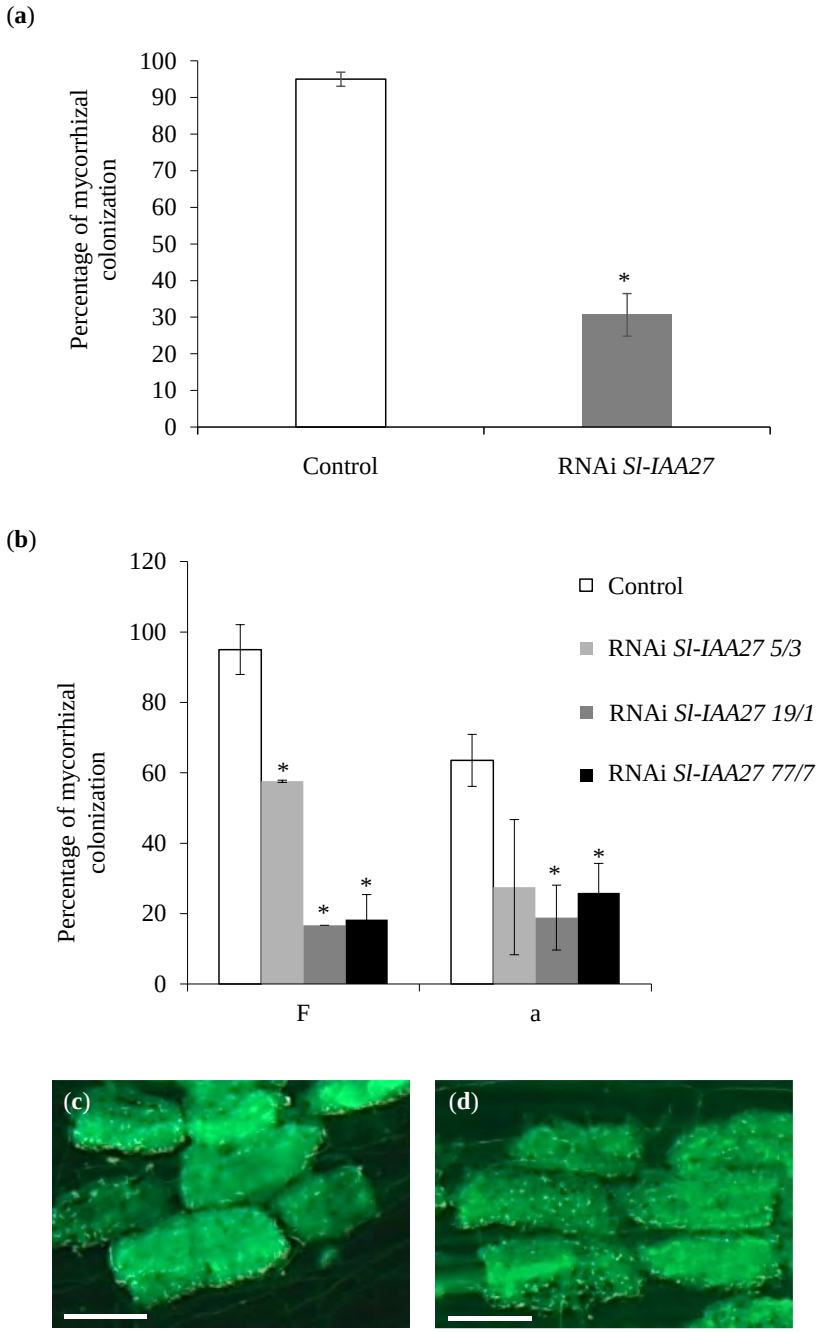

Figure 2: Mycorrhizal phenotype of control and RNAi *Sl-IAA27* lines 12 weeks after inoculation with *R. irregularis*. (a) Mycorrhizal rate in control and RNAi *Sl-IAA27* lines (average of the three lines) as measured by the grid-intersect method (Giovannetti and Mosse, 1980). (b) Quantification of mycorrhization in control and RNAi *Sl-IAA27* lines according to the Trouvelot's method (Trouvelot *et al.*, 1986). 'F': frequency of colonization in the root system; 'a': arbuscule abundance (in percentage) in the colonized root sections. (c, d) Root confocal sections showing arbuscules of control (c) and *Sl-IAA27* RNAi 19/1 roots (d) stained with WGA-FITC. Error bars represent SEM. Stars indicate a significant difference when compared to control according to the Kruskal–Wallis test: n = 10, $p < 0.05$. Scales=50 µm.

2. Results

2.1. *Sl-IAA27* expression is induced by the AM fungus *R. irregularis*

We have previously shown by qRT-PCR that *Sl-IAA27* expression is globally up-regulated in mycorrhized roots of tomato (Bassa *et al.*, 2013). To gain additional information on *Sl-IAA27* expression in roots of mycorrhized plants, we used transgenic *pSL-IAA27::GUS* tomato lines (Bassa *et al.*, 2012). We observed that in non-colonized roots of mycorrhized plants *Sl-IAA27* expression was higher than in roots of non mycorrhized plants (Fig. 1 a, b). In both types of roots GUS expression was higher in young tissues and was mainly localized in the central cylinder and the inner cortex (Figs. 1 a, b, S1 a, b, c). Interestingly, GUS expression was completely absent in the colonized root sections of mycorrhized plants (Fig. 1 d, f). These observations suggest the occurrence of a subtle regulation of *IAA27* expression in mycorrhized roots. While this expression seems to be positively regulated by the general presence of the fungus it is negatively regulated in the immediate vicinity of intraradical fungal structures. To test if diffusible signal compounds released by the fungus are responsible for the positive regulation of *IAA27* transcription, we cultivated *pSl-IAA27::GUS* tomato seedlings (7 day-old) *in vitro* for three days in the presence of germinating fungal spores. Roots and spores were separated by a membrane allowing chemical exchanges but preventing physical contact. The presence of the fungus strongly increased *Sl-IAA27* expression as shown by qRT-PCR (Fig. 1 i) and GUS expression analyses (Fig. 1 g, h), indicating that *Sl-IAA27* up-regulation in mycorrhized roots could be caused by diffusible fungal compound(s).

2.2. *Sl-IAA27* is a positive regulator of mycorrhization

We next investigated whether *Sl-IAA27* plays a role during mycorrhization. We used three independent tomato lines silenced for the expression of *Sl-IAA27* (named RNAi *Sl-IAA27* 5/3, RNAi *Sl-IAA27* 19/1 and RNAi *Sl-IAA27* 77/7, Bassa *et al.*, 2012). Silencing of *Sl-IAA27* results in higher auxin sensitivity and reduced chlorophyll content in leaves. Both ovule and pollen display a dramatic loss of fertility and the internal anatomy of the flower and the fruit are modified (Bassa *et al.*, 2012). As *Sl-IAA27*-RNAi lines were also described to have longer primary roots and higher number of lateral roots when grown *in-vitro* on rich medium (MS/2) (Bassa *et al.*, 2012), we assessed their root architecture in our growth conditions using low phosphate Long Ashton medium. After two weeks in vitro or four weeks in pot, we observed

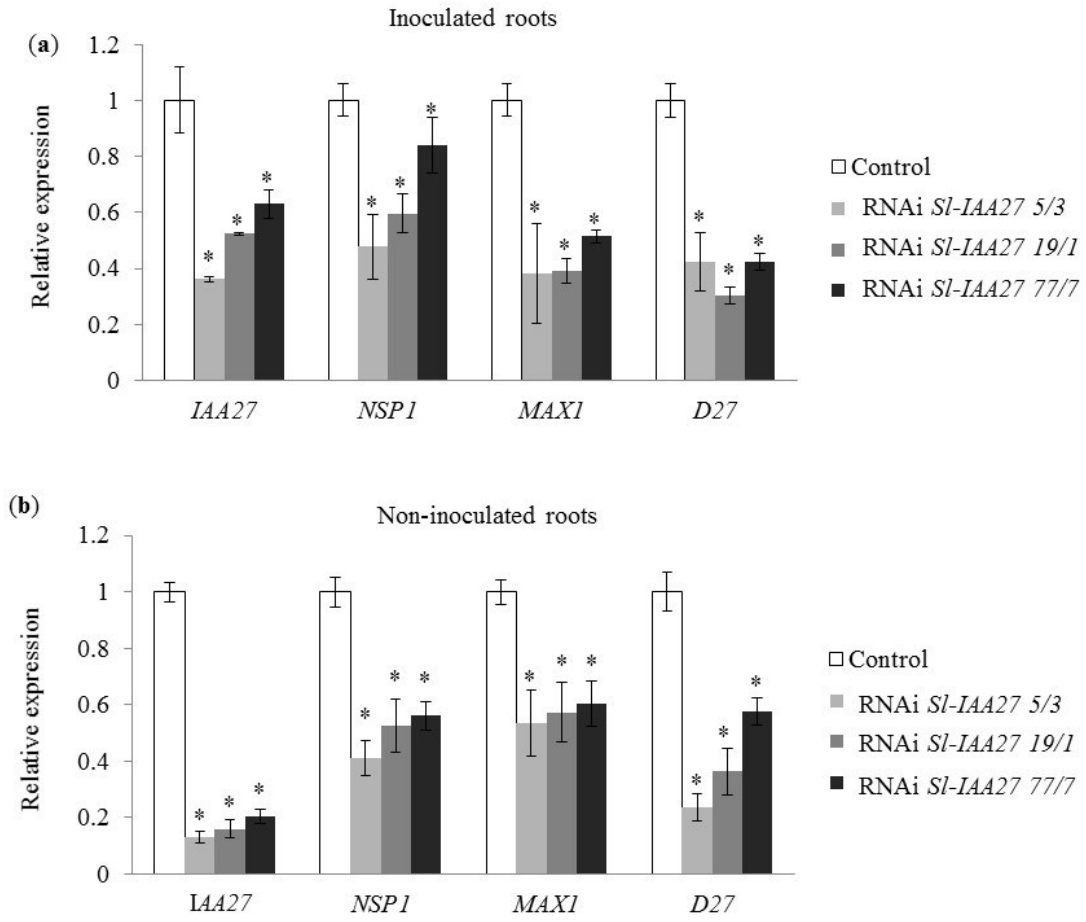

Figure 3: Expression of *Sl-IAA27*, *Sl-NSP1*, *Sl-MAX1* and *Sl-D27* in *Sl-IAA27* silenced lines, with or without mycorrhization. Quantification of *Sl-IAA27*, *Sl-NSP1*, *Sl-MAX1* and *Sl-D27* gene expression by qRT-PCR in inoculated (a) and non-inoculated (b) control and RNAi *Sl-IAA27* plants. Error bars represent SEM. Stars indicate a significant difference when compared to control according to the Kruskal–Wallis test: n = 10 (a), 9 (b), $p < 0.05$.

no differences between the RNAi lines and the wild type, for both the primary root length and the number of lateral roots (Fig. S2). We then inoculated the plants with *R. irregularis* spores, and analyze the root colonization rate 12 weeks after inoculation. Total root colonization was strongly reduced in the RNAi *Sl-IAA27* lines (average of the three lines) compared to the wild type (Fig. 2 a). In agreement with this the expression of the phosphate transporter gene *Sl-PT4*, the tomato homolog of *M. truncatula PT4* (Nagy *et al.*, 2005), which is specifically induced during mycorrhization (Harrison *et al.*, 2002), was also lower in the RNAi *Sl-IAA27* lines (0.4 of the control, data not shown). A closer look to the mycorrhization pattern showed that this lower colonization was due to a strong decrease of the infection frequency and arbuscule abundance (Fig. 2 b). On the other hand the shape and size of arbuscules looked identical in control and *Sl-IAA27*-silenced roots (Fig. 2 c, d). Altogether, these data suggest that *Sl-IAA27* is not involved in the process of arbuscule differentiation but rather in the control of fungal root penetration and intraradical colonization.

2.3. *Sl-IAA27* influences *NSP1* expression

We have previously reported that in *Medicago truncatula* one important GRAS transcription factors of the nodulation process, NSP1, is involved in the control of mycorrhizal root colonization (Delaux *et al.*, 2013). To assess the potential link between *Sl-IAA27* and *NSP1*, we compared the expression of its closer homologous gene in *S. lycopersicum*, in mycorrhized roots of control and RNAi *Sl-IAA27* tomato plants. Only one homologous gene was found by direct blast on *S. lycopersicum* genome (Solyc03g123400.1.1). *Sl-NSP1* expression was down-regulated in *Sl-IAA27*-silenced roots compared to control roots (Fig. 3a). As Liu *et al.* (2011) showed in *M. truncatula* and rice that NSP1 regulates the expression of *D27* and *MAX1*, two genes involved in the SL biosynthetic pathway, we also measured the expression of the closest homolog of these two genes, *Sl-D*27 and *Sl-MAX1*, in mycorrhized tomato (Challis *et al.*, 2013). We found that *Sl-D*27 and *Sl-MAX1*expression was also down-regulated in the three RNAi *Sl-IAA27* tomato lines compared to control plants (Fig. 3a). The same results were obtained in non-mycorrhized plants (Fig. 3b), indicating that the observed down-regulation of *NSP1*, *MAX1* and *D27* in mycorrhizal *Sl-IAA27*-silenced roots was not due to the lower mycorrhization rate.

It has been reported that a close *IAA27* related gene, *AUX/IAA9*, was up-regulated in young fruits of the *Sl-IAA27* RNAi lines (Bassa *et al.*, 2013). Therefore we analyzed the expression of *AUX/IAA9* in non-mycorrhized roots to verify if this up-regulation in fruit was

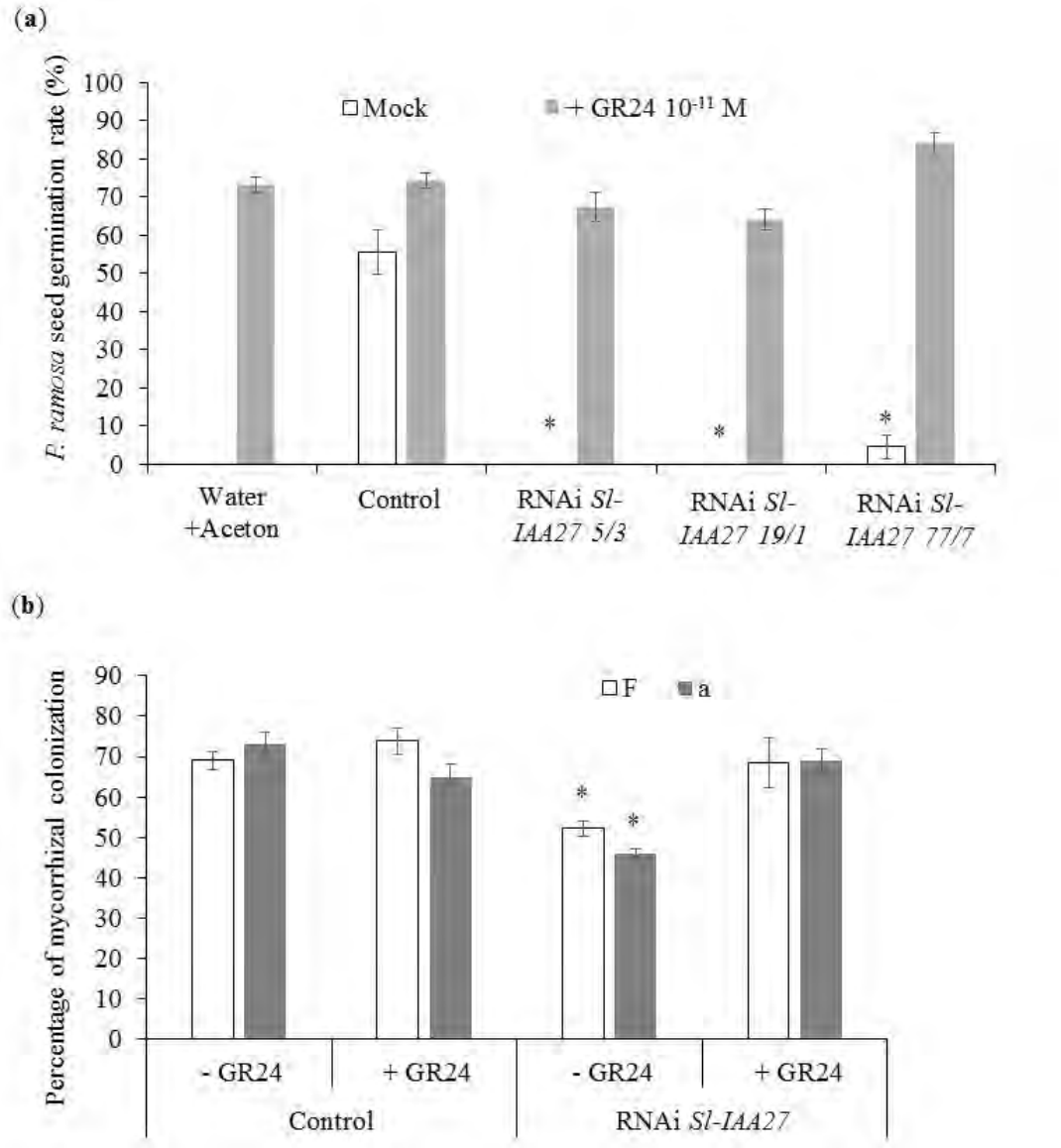

Figure 4: Quantification of strigolactones in roots of control and RNAi *Sl-IAA27* plants, and effect of GR24 on mycorrhization of control and RNAi *Sl-IAA27* plants. (a) Germination rate of seeds of *Phelipanche ramosa* in response to solvent and to root exudates of control and RNAi *Sl-IAA27* plants, with or without addition of 10^{-11} M of synthetic strigolactone (GR24). (b) Percentage of mycorrhizal colonization in roots of control and RNAi *Sl-IAA27* plants with or without 10^{-7} M of GR24, 12 weeks after inoculation, according to the Trouvelot's method (Trouvelot *et al.*, 1986). 'F': frequency of colonization in the root system; 'a': arbuscule abundance (in percentage) in the colonized root sections. Four replicates per RNAi *Sl-IAA27* line were used here and mean values represent the average obtained with the three lines. Error bars represent SEM, stars indicate a significant difference between control and RNAi *Sl-IAA27* plants according to the Kruskal–Wallis test: n=15 $p < 0.01$ (a), n=12, $p < 0.05$ (b).

also effective in roots. We detected no significant changes of *Sl-IAA9* expression in roots of silenced *Sl-IAA27* lines. In addition we assessed in the *Sl-IAA27* RNAi lines the expression of the closest homologous genes of *Sl-IAA27* and *Sl-IAA9*, *Sl-IAA8* (Fig. S3, Audran-Delalande *et al.*, 2012), and we detected no significant changes.

These data suggest that the down-regulation of *NSP1*, *MAX1* and *D27* in *Sl-IAA27* lines were not the result of some indirect *IAA9* and/or *IAA8* regulation. They support the hypothesis that Sl-IAA27, among other yet unknown regulatory roles, could be an Aux/IAA specifically involved in the regulation of *NSP1* expression and therefore indirectly involved in the regulation of SL biosynthesis.

2.4. Mycorrhizal defect of RNAi *Sl-IAA27* lines can be complemented by GR24 addition.

To investigate further the possible role of Sl-IAA27 in the regulation of SL biosynthesis, we performed mass spectrometry analyses of root extracts of wild type and *Sl-IAA27*-silenced plants to compare their SL content. We could not detect the presence of SL in any of those extracts probably because they are in trace amount in *S. lycopersicum* cv. *MicroTom.* Therefore we compared the ability of the extracts to stimulate seed germination of the parasitic plant *Phelipanche ramosa*. This *in vivo* assay has long been used to detect the presence of SL in plant extracts (Dörr *et al.*, 1994; Bouwmeester *et al.*, 2003, Echevarría-Zomeño *et al.*, 2006, Yoneyama *et al.*, 2010, Dor *et al.*, 2011). It can detect SL with a much higher sensitivity (down to 10^{-13} M, Fig. S4) than that of a mass spectrometry analysis (10^{-9} M, V. Puech-Pagès personnal communication), and it provides a better dynamic range for their quantification. As expected, when treated with the control solvent, the germination rate of *P. ramosa* seeds was null, while 73% germination was obtained in the presence of 10^{-11} M GR24 (Fig. 4a). A similar rate of germination (65%) was obtained when seeds were treated with exudates of control roots, whereas none of the seeds germinated when treated with root extracts of RNAi *Sl-IAA27* plants. Moreover, the addition of GR24 (10^{-11} M) to the RNAi *Sl-IAA27* root extract stimulated *P. ramosa* seed germination as efficiently as when added to the solvent or to the control root extract, showing the absence of germination inhibitors in the RNAi *Sl-IAA27* root extracts (Fig. 4a). These results indicate that root extracts of RNAi *Sl-IAA27* plants were at least ten times less active than extracts of control roots (Fig. 4, Fig. S4), therefore suggesting that SL synthesis of RNAi *Sl-IAA27* roots could be strongly down-regulated.

To ask whether the mycorrhizal deficiency of the RNAi *Sl-IAA27* plants could result

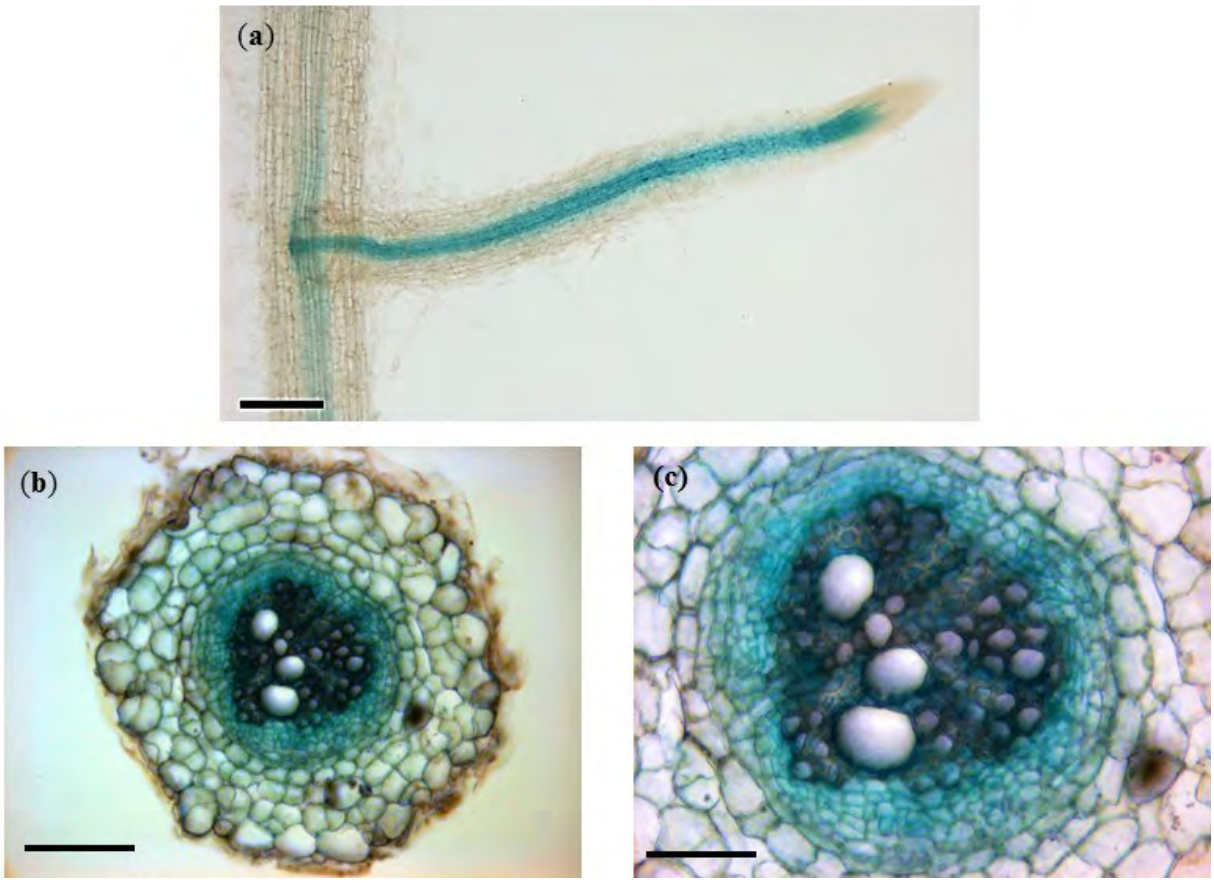

Figure S1: Localization of *Sl-IAA27* expression in roots of 4 week-old non mycorrhized tomato plants. (a) Strong expression of the *pSl-IAA27::GUS* construct in young emerged lateral roots. (b) and (c) same transversal section at two magnifications showing that this expression is mainly in the central cylinder and the inner cortex. Similar GUS expression was found in the non-colonized root sections of mycorrhized plants. Scale bars (a) = 200 µm, (b) = 100 µm, (c) = 50 µm.

from this SL down-regulation, we performed a mycorrhization assay with control and *Sl-IAA27*-silenced plants in the presence or not of 10^{-7} M GR24. The addition of the synthetic SL complemented the mycorrhizal defect of RNAi *Sl-IAA27* plants, especially by increasing the infection frequency as well as arbuscule abundance (Fig. 4 b), strongly suggesting that the mycorrhizal defect of these plants was due to a lower SL biosynthesis.

3. Discussion

Here we collected several experimental evidences suggesting that the auxin-related gene *Sl-IAA27* positively regulates the mycorrhization process of tomato by controling the strigolactone synthesis via direct or indirect regulation of *NSP1*, a transcription factor which activates the SL biosynthesis genes *D27* and *MAX1* (Liu *et al.*, 2011). Indeed, we showed that the mycorrhizal defect of *Sl-IAA27*-silenced plants was correlated to a down regulation of *NSP1*, *D27* and *MAX1* expression and arguably to a lower SL content in roots, which could be complemented by exogenous GR24 treatments.

We present a first demonstration of the importance of an Aux/IAA in the regulation of SL biosynthesis showing an additional cross-talk link between auxin and SL (Foo, 2013; Koltai, 2015). Given that Aux/IAAs are known to interact with ARF partner proteins, preventing them from binding to target promoters, we can speculate that *Sl*-IAA27 represses an ARF that acts as a repressor of *NSP1* expression. This repressor ARF remains to be identified, and it would be interesting to check the occurrence of this regulation in non-mycotrophic species, such as *Arabidopsis thaliana*.

Interestingly, we found that *Sl-IAA27* expression is induced by the fungus very early in the mycorrhizal interaction, even before any root-fungus physical contact. We hypothesize that this induction is caused by some diffusible fungal signals and may result in enhanced SL synthesis in roots. This would lead to an increase of SL content in root exudates and to the activation of the fungus metabolism in the rhizosphere (Besserer *et al.*, 2006; 2008). In the root the presence of the fungus would switch off the transcription of *Sl-IAA27* locally while up-regulating this expression remotely in not yet colonized root tissue. Further investigation will be needed to determine if this *IAA27* transcriptional activation is due to intra- and/or extra-radical diffusible fungal signals and what role this activation could have in the mycorrhization process. The local down-regulation of *Sl-IAA27* transcription in colonized root tissue is reminiscent of previous observation of a strong localized activation of

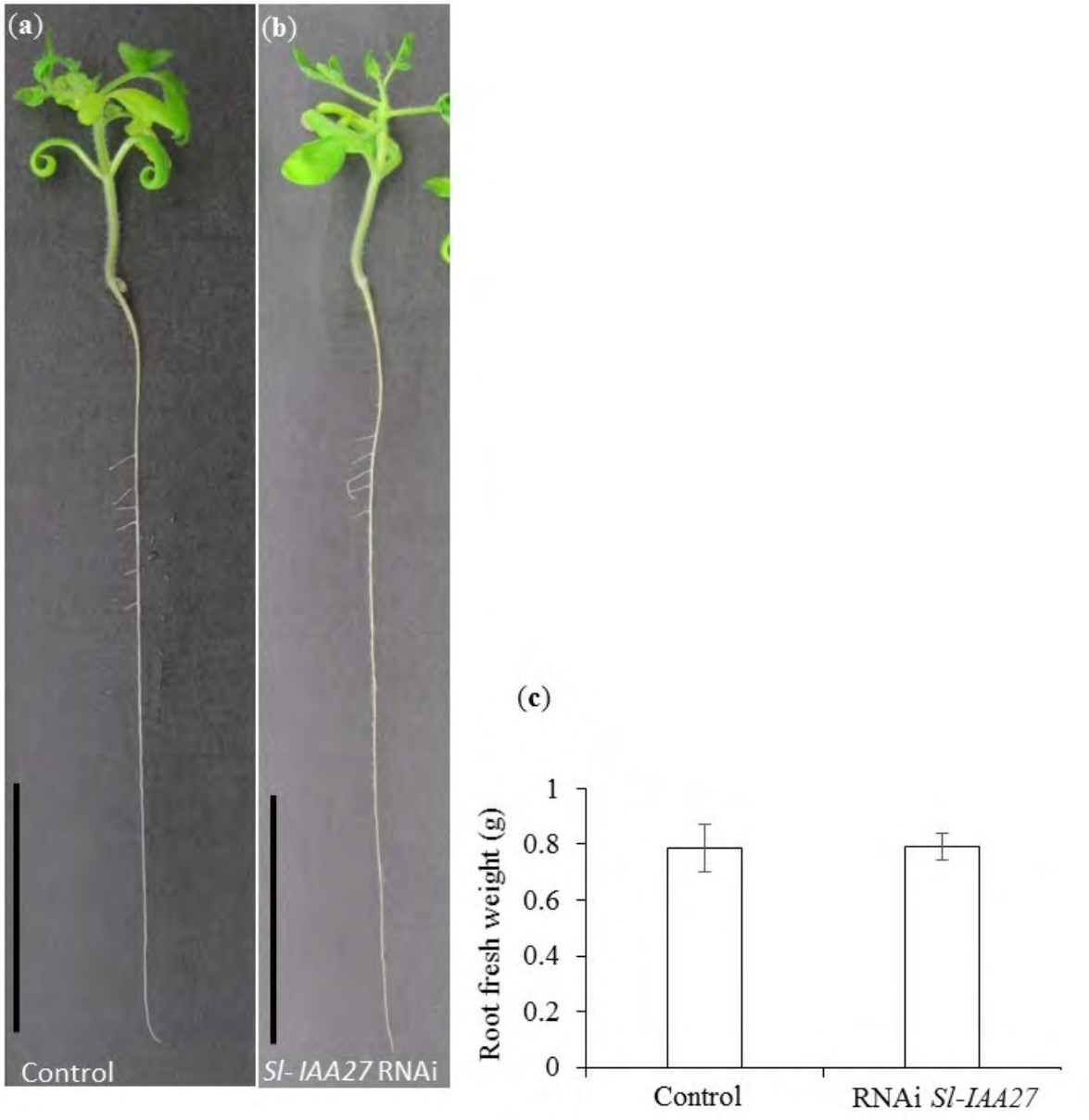

Figure S2: (a, b) Root architecture of 2 week-old *in-vitro* control (a) and RNAI *Sl-IAA27* (b) tomato plants grown in low phosphate Long Ashton, scale bar = 5cm. (c) Fresh root weight of four week-old tomato plants grown in pots and watered with low phosphate Long Ashton, n=10, error bars represent SEM.

DR5-GUS, an auxin-reporter construct, in arbuscule-containing cells (Etemadi *et al.*, 2014). Given that *Sl-IAA27* expression of tomato can be down-regulated by treatment with exogenous auxin, at least in 12 day-old seedlings (Bassa *et al.*, 2012), the lack of *Sl-IAA27* expression in the inner cortex and the vascular tissue of colonized root sections might be due to an activation of auxin signaling in neighboring tissues.

We assume that a clear difference has to be made between the early colonization stages, when fungal growth has to be stimulated, and the later stages of colonization, when mycorrhization and trophic exchanges have to be tightly controlled and balanced (notably to minimize the carbon cost for the host plant, Peng *et al.*, 1993). During this late colonization stages when the plant is well colonized, it is commonly known that SL content in roots decreases (López-ráez *et al.*, 2011), while auxin content increases (reviewed in **Fusconi, 2014**). Here we speculate that the auxin-mediated down-regulation of *Sl-IAA27* transcription in colonized root sections, by negatively regulating *NSP1* expression and SL synthesis, participates to the complex process of auto-regulation of mycorrhization and perhaps also to the process of arbuscule degeneration.

The present study illustrates the importance of careful spatiotemporal analyses for understanding the regulation mechanisms underlying the complex developmental process of mycorrhization. Further studies are necessary in order to fully understand why and how *Sl-IAA27* expression is regulated at different stages of the mycorrhization process.

4. Materials and Methods

4.1. Plant and fungal materials, growth and conditions

Seeds of tomato (*Solanum lycopersicum* cv. *MicroTom*) wild type, *pSl-IAA27*: GUS lines (three independent lines 14/6/1, 10/10/1 and 37/4/1) and RNAi *Sl-IAA27* (three independent lines 5/3, 19/1, 77/7) were obtained as already described (Bassa *et al.*, 2012). Seeds of the parasitic plant *Phelipanche ramosa* L. Pomel (genetic type 1, Voisin *et al.*, 2011) were provided by P. Simier (LBPV, University of Nantes, France).

Tomato seeds were surface sterilized for 1 min in 2.3 % sodium hypochlorite and washed eight times with sterile deionized water. They germinated on solid water agar plate in the dark at 23°C for 6 days.

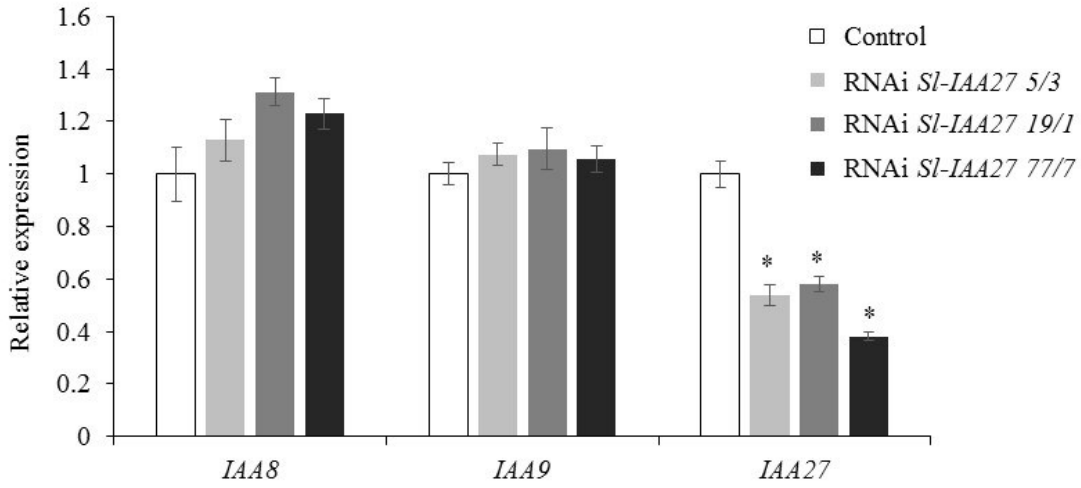

Figure S3: Relative expression of *Sl-IAA8*, *Sl-IAA9* and *Sl-IAA27* as measured by RT-qPCR in 4 week-old control and *Sl-IAA27* tomato plants (error bars represent SEM, stars indicate a significant difference between control and RNAi *Sl-IAA27* plants according to the Kruskal–Wallis test ($n=10$, $p < 0.05$).

For mycorrhization assays and qRT-PCR analyses, seedlings were grown in 250 mL pots (one seedling per pot) filled with Oil-Dri US special substrate (Damolin) for 12 weeks, in a growth chamber (16/8 h day/night, 24°C/22°C, 120-150 µmol $m^{-2}s^{-1}$) and watered every 2 days with modified Long Ashton medium containing a low concentration (7.5 µM) of phosphate (Balzergue *et al.,* 2011). They were inoculated with *Rhizophagus irregularis* DAOM 197198 sterile spores (400 spores per liter of substrate) purchased from Agronutrition (Carbone, France). For GUS staining experiments seedlings were inoculated with a higher inoculum (2000 spores per liter of substrate) and harvested 4 weeks after inoculation.

For in vitro culture, germinated seedlings were grown on solidified modified Long Ashton medium (7.5 µM of phosphate), 8% agar (KALYS BIOTECH, AGAR HP 696) in 12 cm square plates (5 seedlings per plate) in a growth chamber (16/8h day/night, 24°C/22°C, 60 µmol $m^{-2}s^{-1}$). After six days, a cellophane membrane (couvre confiture HUTCHINSON) covered with 500 *R. irregularis* germinating spores was laid on seedling roots for three more days, so that the membrane prevented physical contact, but not the chemical communications between the partners. Prior to this step, the spores had been incubated on the cellophane membrane laid on the same solid modified Long Ashton medium for 6 days at 30°C and 2% CO_2.

4.2. Strigolactone treatment

The SL analog GR24 was purchased from Chiralix B.V. (Nijmegen, The Netherlands). For *P. ramosa* seed germination tests (see below) 10^{-8} to 10^{-13} M water solutions of SLs were prepared from a 10^{-3} M stock solution in acetone. For treatment of tomato plants grown in pots 10^{-7} M GR24 was dissolved in the low phosphate Long Ashton and watered (10 ml/pot) three times a week. Control plants were watered with 0.0001% (v/v) acetone. To minimize the amount of used GR24 twelve plants for control and four plants per RNAi *Sl-IAA27* line were used. For the RNAi *Sl-IAA27* lines, the mean values of Fig. 4b represent the average obtained with the three lines.

4.3. Gene expression analyses*:*

For quantitative RT-PCR analyses, total RNA was extracted using a Plant RNeasy Mini kit (Qiagen) according to the manufacturer's instructions. Total RNA was treated by DNase I (Promega) to remove genomic DNA contamination. Reverse transcription was performed using M-MLV Reverse Transcriptase, RNase H Minus, Point Mutant (Promega)

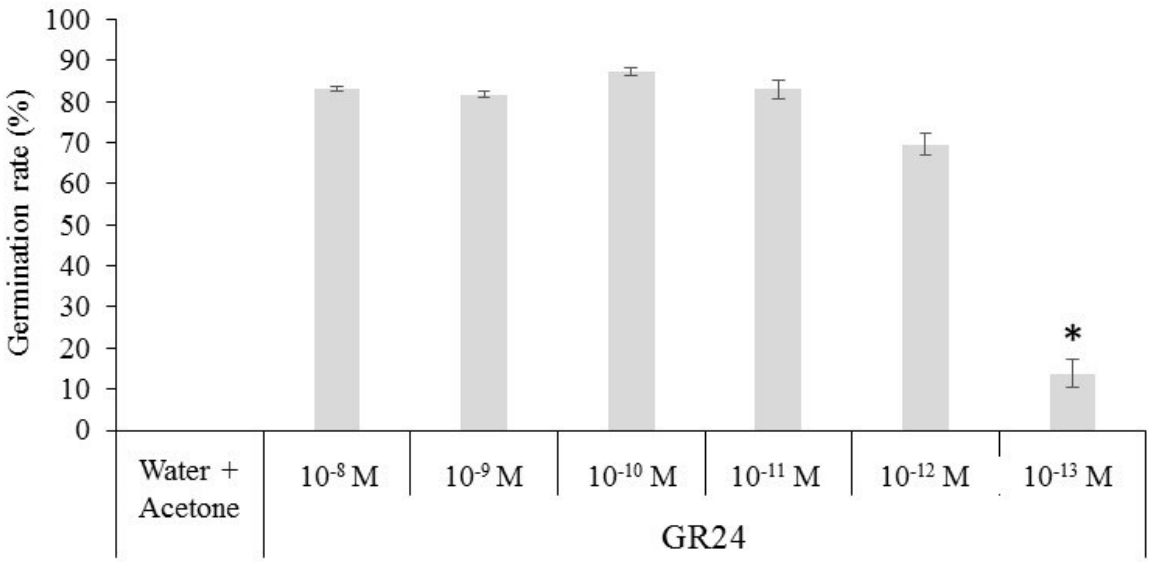

Figure S4: Germination rate of seeds of *Phelipanche ramosa* in response to solvent and to a range of concentrations of synthetic strigolactones (GR24). Error bars represent SEM, star indicates a significant difference between positive control (GR24 10^{-8}M) and the other GR24 concentrations according to the Kruskal–Wallis test ($n=8$, $p < 0.05$).

on 1 µg of total plant RNA. For each experiment, six to twelve independent plants were analyzed. Quantitative PCR amplifications were conducted on a Roche LightCycler 480 System (Roche Diagnostics) under the following conditions: 95°C for 5 min, then 45 cycles of 95°C for 15 sec and 60°C for 1 min. The various primer sets used are described in Table S1. The measured transcripts were normalized by using the Sl-Actin gene.

For histochemical GUS analysis, root tissues of *pSL-IAA27::GUS* tomato lines were soaked in GUS staining solution (100 mM sodium phosphate buffer, pH 7.2, 10 mM EDTA, 0.1 % Triton X-100, 0.3 mg ml^{-1} X-Gluc) under vacuum for 15 min. Root tissues were then incubated 6 to 12 h in GUS staining solution at 37 °C. GUS pictures shown Fig. 1a, b, h, i and Fig. S1 represent a staining pattern found in all *pSl-IAA27*: GUS lines. For GUS pictures shown Fig. 1 c, d, e, f roots were stained as described above, then cleared with KOH and stained with WGA-FITC as described below for mycorrhizal phenotyping.

For transversal root sections, root tissues after GUS staining were included in low melting 5% agar and cut into 50 µm section using vibratom, prior to be observed under stereomicroscope Axio Zoom V16 Zeiss.

4.4. Mycorrhizal phenotyping and fungus staining

Roots were cleared in 10% w/v KOH for 8 min at 95°C and rinsed in sterile water. Then they were treated for 30 min with fluorescein-conjugated wheat germ agglutinin (WGA-FITC) (Invitrogen), which binds fungal chitin, washed three times for 10 min in PBS and observed using a stereomicroscope Axio Zoom V16 Zeiss. Arbuscular size and shape has been analyzed by using confocal microscope LEICA TCS SP8. Alternatively, roots were stained with Schaeffer black ink as described by Vierheilig *et al.* (1998). The percentage of mycorrhization was established using the grid intersect method described by Giovannetti and Mosse (1980) and with two additional mycorrhization indices: F, mycorrhization frequency and a, arbuscule abundance in colonized root sections, according to Trouvelot *et al.* (1986).

4.5. Statistical analyses

Means were calculated with values of 6 to 15 replicates ($n < 25$) depending on the experiments (indicated in figure legends) and therefore were compared by using the Kruskal–Wallis test. Each experiment was repeated two to three times.

4.6. P. ramosa germination assay

Root extracts: One gram of powdered N_2-frozen roots of tomato (8 week-old) grown in pots as described above was suspended in 2 ml of 100 % ethyl acetate and sonicated for 10 min in 4°C water. After centrifugation at 2000 rpm for 10 min at 4 °C, the upper organic phase was transferred into new tube and the extraction of the pellet was repeated with 2 ml of fresh ethyl acetate. The two organic phases were pooled prior to be washed with 0.2 M K_2HPO_4 buffer and then dried under nitrogen flow. Root extracts used to stimulate germination of *P. ramosa* seeds were resuspended in 25 % acetone and diluted 1000 to 100 000 times in sterile deionized water before use.

P. ramosa seeds were surface-sterilized by vigorous agitation in a 2.3 % sodium hypochlorite solution for 5 min. They were then washed with sterilized deionized water three times for 30 s and three times for 5 min, and then they were transferred for 10 days in the dark at 24°C in 12 well-plates (approximately 300 seeds per well) containing 0.5 ml of sterilized deionized water per well. After this preconditioning period, water was removed and replaced by 0.5 ml of diluted root extract (see § above). After 7 days seeds were stained with 0.5 % neutral red w/v and germination rate was assessed under a stereomicroscope Leica MZ75.

Acknowledgements

This work was funded by the French ANR project miRcorrhiza (ANR-12-JSV7-0002-01). It was carried out in the *LRSV* which belongs to the *Laboratoire d'Excellence* entitled TULIP (ANR-10-LABX-41). We thank P. Simier (LBPV, University of Nantes, France) for providing seeds of *Phelipanche ramosa* L. Pomel (genetic type 1) and V. Puech-Pagès (LRSV, Castanet-Tolosan, France) for the MS analyses. We also thank A. Le Ru (Genotoul, FRAIB 3450 CNR, Castanet-Tolosan, France) for her help with confocal microscopy.

Author contributions

J.-P.C., C.A., M.B., G.B. planned and designed the research, B.G, M.E. performed experiments, J.-P.C., B.G., G.B. wrote the manuscript.

Discussion and Conclusion

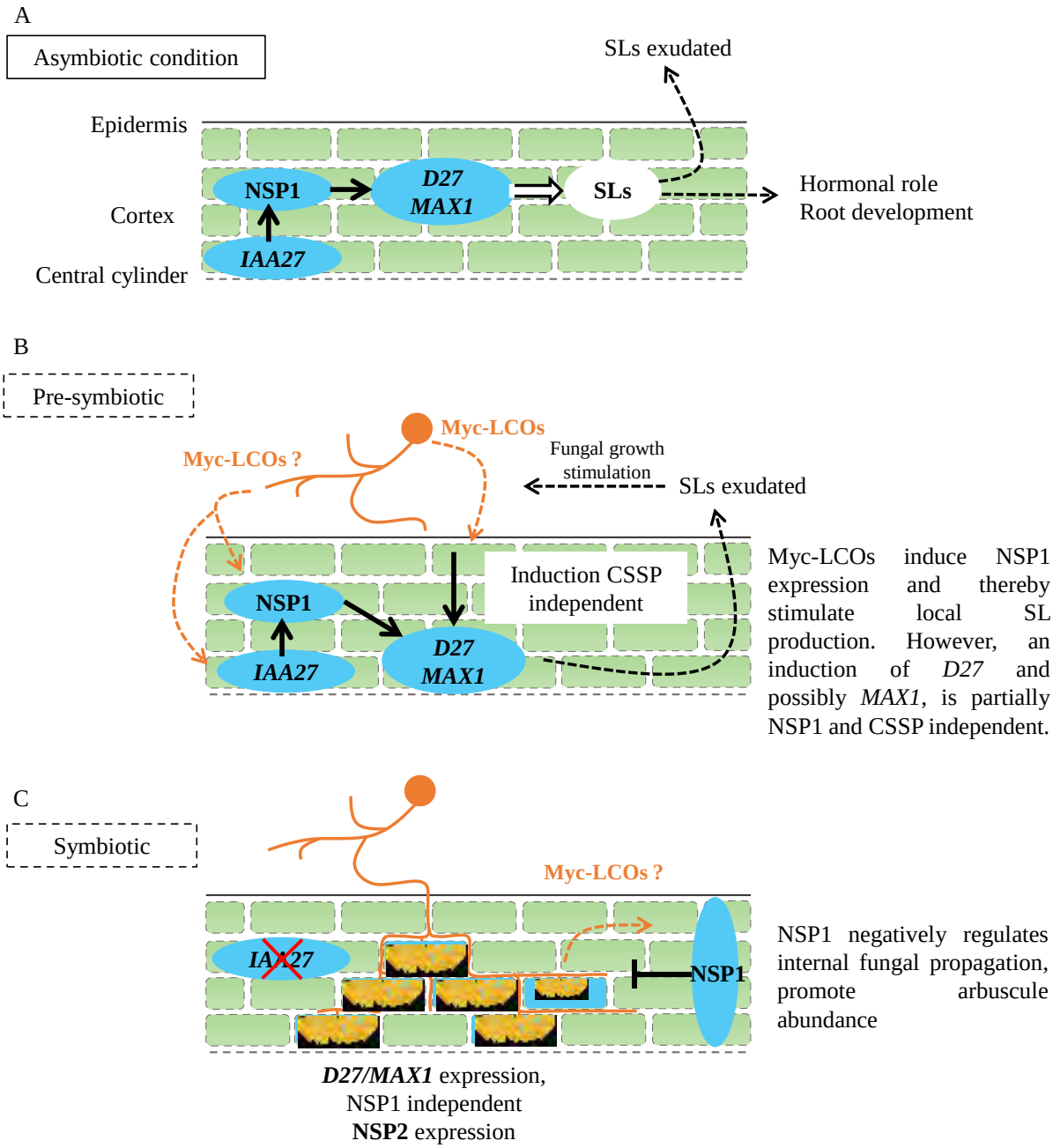

Figure 1: Schematic representation of the involvement of *IAA27*, NSP1, *D27* and *MAX1* during the different steps of fungal infection. (A) In asymbiotic conditions, NSP1 is required for the induction of *MtD27* and *MtMAX1* that produced SLs. At this stages, SLs might have an hormonal role in root development, but also be exudated into the rhizosphere. The expression of *Sl*-NSP1, *Sl-D27* and *Sl-MAX1* is promoted by *Sl-IAA27*. (B) In pre-symbiotic condition, NSP1 (still promoted by *IAA27*) plays a major role in the Myc-LCOs mediated induction of *D27* and *MAX1*. However, a minor part of this induction is also NSP1, and CSSP independent. This induction might result in a local increase of SLs exudation that stimulate the AMF in the rhizosphere. (C) When the fungus has entered the roots, the induction of *D27* and *MAX1*, localized in the fungal containing structures, is NSP1 independent. NSP1 is then expressed in the "going to be colonized cells" and seems to repress fungal propagation, and promote arbuscule formation. This local induction of NSP1 might be due to Myc-LCOs. At this stage NSP2 is expressed while *Sl-IAA27* is not induced in the colonized root part.

1. Discussion

Resulting from an extremely long plant-fungus co-evolution, the mycorrhization process is expected to be regulated by a complex molecular regulatory network. Here, by focusing on only two transcription factor-encoding genes, *NSP1* and *NSP2*, recently found to be involved in the mycorrhization process, we provided additional evidence that the regulation of mycorrhizal colonization is highly spatio-temporal and integrated. We unraveled three distinct mechanisms, a SL-dependent one, a SL-independent one and a third one involving an original target mimicry process.

1.1. Roles of SLs in asymbiotic condition.

First of all, we could confirm that *Mt*NSP1 is required for the direct induction of *MtD27* and *MtMAX1* leading to the production of SLs. At this stage, because these three genes are mostly expressed in the meristematic root parts, SLs might have a hormonal role in root development. We also know that SLs can be exudated into the rhizosphere, especially in phosphate starvation. During this asymbiotic condition, the expression of *Sl*-NSP1, *Sl-D27* and *Sl-MAX1* is promoted by *Sl-IAA27* (Fig. 1 A). Whether or not the induction of *NSP1* expression by Myc-LCOs in *M. truncatula* is also dependent on an IAA27 orthologue, is an open question.

1.2. Role of SLs in the early step of mycorrhizal colonization

We propose that during the very early steps of fungal colonization, when the fungus is still in the rhizosphere, Myc-LCOs induce the expression of *MtNSP1*, and thereby *MtD27*/*MtMAX1*, leading to the production of SLs crucial for an effective fungal entrance in the roots (Fig. 1 B). As shown in tomato the *NSP1* expression requires the presence of an AUX/IAA, *Sl-IAA27*, which is also induced by Myc-LCOs.

1.3. Regulation of SL biosynthesis genes in colonized tissues

Later on, when the fungus has reached the inner cortical tissue of the roots, a totally different pattern of regulation seems to occur (Fig. 1C). First of all, *Sl-IAA27* expression, induced in mycorrhizal roots (Chapter 3), is excluded from the arbuscule-containing cells of tomato. We hypothesized that this down-regulation results from the intense auxin signaling that takes place in these cells (Etemadi *et al.*, 2014). In *M. truncatula* the expression of NSP1 follows the same pattern: it is also generally induced in mycorrhizal roots but specifically absent in the colonized root sections. This parallelism suggests that NSP1 and IAA27 could belong to

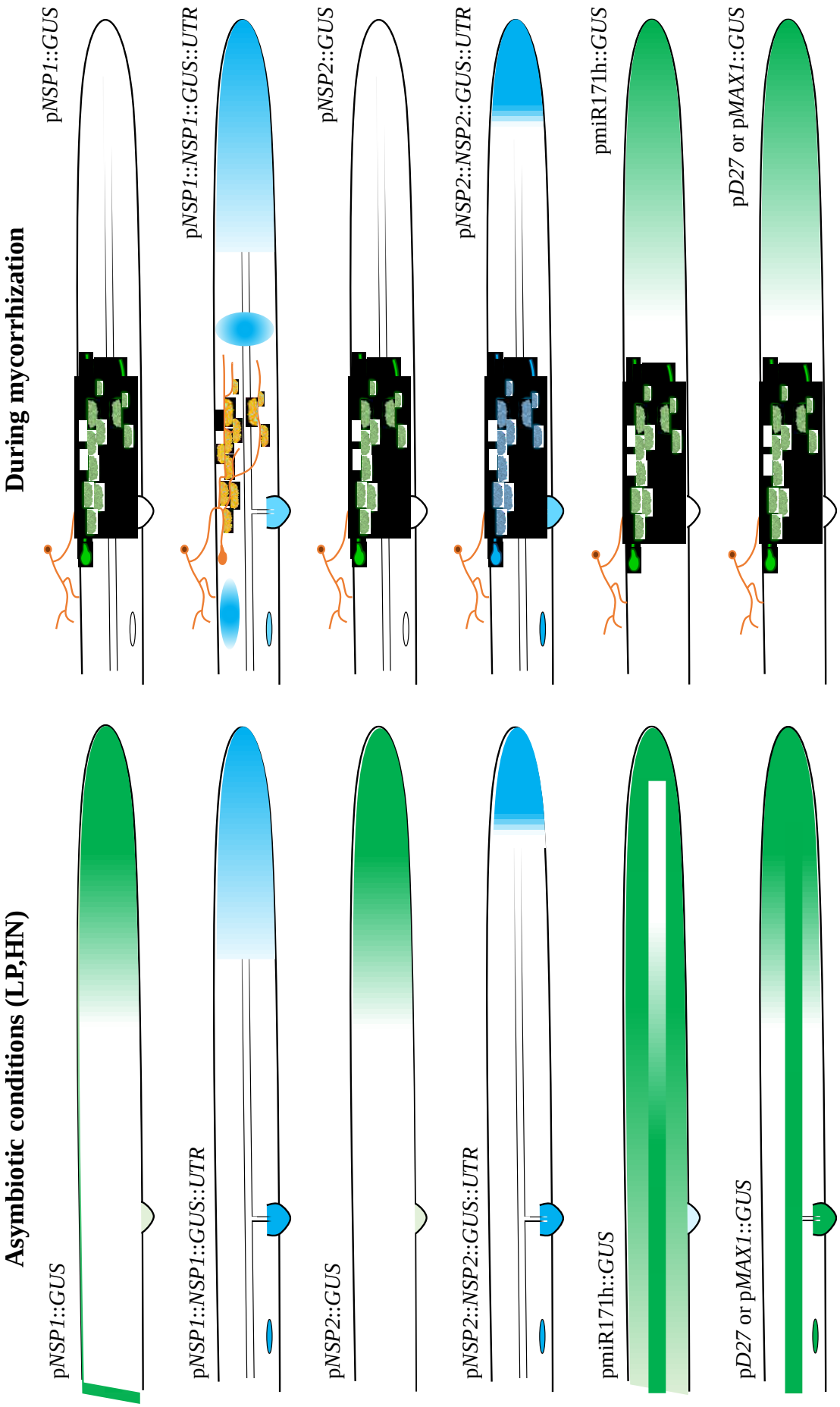
During mycorrhization
pNSP1::GUS
pNSP1::NSP1::GUS::UTR
pNSP2::GUS
pNSP2::NSP2::GUS::UTR
pmiR171h::GUS
pD27 or pMAX1::GUS
Asymbiotic conditions (LP,HN)
pNSP1::GUS
pNSP1::NSP1::GUS::UTR
pNSP2::GUS
pNSP2::NSP2::GUS::UTR
pmiR171h::GUS
pD27 or pMAX1::GUS

Figure 2

the same signaling pathway both in tomato and in *M. truncatula*. Surprisingly, whereas the transcription of the two SL biosynthesis genes *D27* and *MAX1* are known to be regulated by NSP1 (Liu *et al.*, 2011; Chapter 1), we found that their expression is highly up-regulated in the colonized root tissues, where NSP1 is not expressed, strongly suggesting that in the late stages of colonization their transcription is no longer dependent on NSP1. It could be dependent on other(s), yet unknown, transcription factor(s) specifically occurring in arbuscule-containing cells. The role of NSP2 on *D27* and *MAX1* transcriptions is still to be established more precisely. Liu *et al.*, in 2011 showed that the a-symbiotic expression of *D27* and *MAX1* was also greatly dependent on NSP2. It would be important to determine if NSP2, with or without other proteic partners, is required for *D27* and *MAX1* expression, not only in the a-symbiotic conditions but also in arbuscule-containing tissues.

1.4. Deeper insight into the role of NSP1 and NSP2 in the mycorrhizal colonization

We showed that NSP1 and NSP2 proteins were colocalized in the root apical regions and in the nodules (Chapter 1, Fig. S1). From this observation, we can speculate that NSP2 and NSP1 act within the same proteic complex to fulfill their regulation of root development and nodule formation. Moreover, as NSP1 and NSP2 are essential for nodule formation and as SLs have not been found to be crucial for nodule morphogenesis (Foo & Davies, 2011; Liu *et al.*, 2013; Foo *et al.*, 2013), the roles of NSP1 and NSP2 might be SL independent in this specific organogenesis. And consistent with their very specific expression in the meristematic cells we could think that during evolution rhizobial bacteria might rely on the meristematic properties of theses TFs to induce the formation of nodules.

Interestingly, during the late stages of mycorrhization, NSP1 and NSP2 proteins are not colocalized anymore, suggesting that, here, they have different functions (Fig. 2 blue).

Figure 2: Schematic representation of the different GUS expression pattern of NSP1, NSP2, *miR171h*, *D27* and *MAX1*. In green are represented the transcriptional fusions, and in blue the translational fusions. Transcriptional fusion of p*NSP1*, p*NSP1* and p*miR171h* have been done in the lab and confirm the expression pattern published in Untergasser *et al.*, 2012, Laurressergues *et al.*, 2012, Hofferek *et al.*, 2014. On the left is represented the GUS expression pattern in asymbiotic conditions, and on the left the respective GUS expression in mycorrhizal condition. In orange is represented the fungus.

In agreement with this, the mycorrhizal phenotypes of *nsp1* and *nsp2* are different. While NSP1 and NSP2 seem to be necessary for efficient arbuscule formation, NSP1 appears to repress the propagation of intraradical hyphae, whereas NSP2 seems to promote this hyphal extension. In addition, even if both *nsp1* and *nsp2* display a reduced abundance of arbuscule, this reduction is less severe *in nsp2* (Chapter 1 Fig. 8C). As the phenotype of the double mutant *nsp1/nsp2* resembles of the *nsp1* mutant (higher intraradical hyphal extension), the *nsp1* phenotype could be "dominant". Given that i) GR24 treatment failed to restore a wild-type phenotype in the *nsp1* mutant (no reduction of hyphal propagation within the roots and no increase of the arbuscule abundance), and ii) NSP1 presence did not co-localize with those of *D27* and *MAX1*, we conclude that the control of the mycorrhizal colonization by NSP1 does not rely on its control of SL synthesis (Chapter 1 Fig. 9 B, C).

Because the NSP1 transcription factor is very locally expressed in the cells just above the fungal colonization front, we could speculate that in these cells, NSP1 may induce the transcription of several target genes, preparing the cells for efficient arbuscule formation. The structural reorganization in the not yet colonized cells at the vicinity of the fungus has been already described by Genre *et al.*, 2008, and NSP1 might be one of these early induced factors (by Myc-LCOs?). The absence of NSP1 would lead to a failure in cell reorganization and priming, resulting to a lower number of arbuscule formed in the *nsp1* mutant.

Finally as discussed in the chapter 1, during mycorrhization NSP2 and NSP1 would not interact, and because of its structure NSP2 could interact with several other GRAS transcription factors, improving their efficiency for the regulation of a large set of target genes leading to an efficient formation of arbuscules.

1.5. Role of SLs in the formation of arbuscules?

Understanding the specific role of SLs during the different steps of the colonization process is particularly difficult because any perturbation of SL biosynthesis would lead to an undecipherable phenotype resulting from the combination of SL various early and late effects. In addition, as SL biosynthesis genes are also expressed in the apical root parts, there is a risk that a mutation of these genes would have some consequence on root development with possible indirect effect on fungal colonization.

Anyhow it remains to know why SL biosynthesis genes are expressed in the colonized cells and what would be their roles? As auxin signaling was found to be crucial for the differentiation of arbuscules (Etemadi *et al.*, 2014), we hypothesize that one SL function

could be to influence auxin diffusion in the colonized cells via PIN relocalization (Koltai *et al.*, 2010a; Ruyter-Spira *et al.*, 2011). Moreover SLs have been related to TCP transcriptional factors (modulators of plant growth and development, Li 2015) and also to cytokinins (CKs) that could play a role during mycorrhization (Minakuchi *et al.*, 2010; Braun *et al.*, 2012; Mason *et al.*, 2014; Rameau *et al.*, 2015). Taking into account that the CK content increases in the roots during mycorrhization and that treatments with exogenous CKs increase mycorrhization (Allen *et al.*, 1980; van Rhijn *et al.*, 1997; Ginzberg *et al.*, 1998; Laffont *et al.*, 2015), we speculate that a cross talk between auxin, SLs and CKs must take place for controlling the arbuscule development. In addition, *NSP2* expression has been related to the CK pathway and the common symbiotic pathway. Indeed the Nod factor-mediated induction of *NSP2* was impaired in the CK insensitive mutant *cre1* (Heckmann *et al.*, 2011; Ariel *et al.*, 2012). Furthermore gibberellins have also been shown to repress the *NSP2* induction by Nod factors (Maekawa *et al.*, 2009), illustrating the complex cross talk that takes place between all these phytohormones during the mycorrhizal symbiosis.

In an attempt (data not shown) to decipher the role of SLs in the colonized root tissues, where we observed a very local expression of *D27* (Chapter 1), we created different siRNA constructs against *D27*. To silence *D27* only when the fungus has entered the root, we made an siRNA cassette under the regulation of the vapyrin promoter (Pumplin *et al.*, 2010). In the three biological repeats, the local silencing of *D27* in the colonized tissues, showed either a better, a lower or an equal colonization rate compared to the control (data not shown). These results suggest that the involvement of SLs during the later steps of colonization is not very strong. Or it also might be due to the fact that the RNAi silencing of *D27* is not always 100%. The remaining *D27* transcription might have been sufficient for some SL biosynthesis and induction of SL signaling in the arbuscule-containing cells resulting in a weakly visible phenotype. However, this study should be pursued with the use of other promoters to drive the expression of the *D27* RNAi cassette. For example the use of the promoter of the *MtPt4* gene, a phosphate transporter only expressed in cells containing fully functional arbuscule (Harrison *et al.*, 2002), would be interesting. In complement, the use of promoters with an expression apart from the arbuscule-containing cells would be very interesting too.

In rice, the *d3* and the *hebiba* mutant affected in a gene encoding for an F-box protein and an α/β hydrolase respectively, crucial for SL signaling, displayed a strong mycorrhizal

defect with aborted infection entrance, and hence no arbuscule formation (Yoshida *et al.*, 2012; Gutjahr *et al.*, 2015). In order to fully understand the impact of SLs on the different steps of the mycorrhization process, the study of a more complete collection of biosynthesis and signaling mutants will have to be carried out.

1.6. Discovery of a new mechanism of regulation of *NSP2*

Finally, the discovery of the possible implication of *NSP1* in the promotion of *NSP2* expression, via a target mimicry effect of *NSP1* RNA messenger on miR171h, represents an additional layer of complexity. Indeed, given the fact that *NSP1* transcripts are present (Chapter 2) but NSP1 proteins are absent (Chapter 1) in the arbuscule-containing cells, we can speculate that the presence of these transcripts in these cells is solely for their miR171 mimic function and therefore for promoting the expression of *NSP2* (Fig. 2). It is possible that the relative abundance of the three types of molecules, *NSP1* mRNA, miR171h and *NSP2* transcipts varies in colonized cells and in non-colonized tissues nearby, leading to some differential *NSP2* expression and consequently to a subtle spatio-temporal tuning of genes involved in the dynamic of mycorrhizal colonization (Benkovics & Timmermans, 2014).

The differential expression pattern of NSP1 during mycorrhization, where *NSP1* transcripts, but not the NSP1 proteins, are present in the colonized tissues, leads to additional hypotheses. Thus we could hypothesize that *NSP1* is transcribed in colonized cells where it is either not translated, or translated and rapidly degraded. Because we observed a basal level of transcription throughout the roots in asymbiotic conditions (Chapter 2, Fig. S2), it is possible that the accumulation of NSP1 in the not yet colonized tissues was due to a local translation and/or stabilization of the protein. Interestingly, *MtNSP1* but also the rice *NSP1* (AC135559) are close homologous genes of the *A. thaliana* GRAS transcriptional factor SHORT-ROOT (SHR) (Tian *et al.*, 2004; Xue *et al.*, 2015) which is transcribed in one cell type while the protein accumulates in another tissue (Nakajima *et al.*, 2001). Similarly, NSP1 might have a similar capacity of migration: it would be synthesized in the colonized tissues from which it would be exported toward the colonization front.

2. Conclusion

We brought new knowledge regarding the role of *Mt*NSP1 in the regulation of mycorrhization. During the early phase of mycorrhization the role of *Mt*NSP1 would be crucial for the positive transcriptional regulation of *MtD27* and *MtMAX1*. This would presumably stimulate SL biosynthesis, the fungal growth and the frequency of the infection sites. Then, *Mt*NSP1 would not be involved in the regulation of these two genes for the colonization process, and another regulatory pathway, not dependent on NSP1, would take place. During this stage, *Mt*NSP1 would rather play a role as a negative regulator of fungal propagation in the root, and as a positive regulator of arbuscule formation. These regulatory activities would be performed in cells close to a mycorrhizal root zone, prior to be colonized. In the colonized zones however, *MtNSP1* transcripts would still be present. There, they would play an unexpected and original role. They would promote *MtNSP2* expression, by buffering the negative action of miR171h, a microRNA of *M. truncatula* that targets *MtNSP2*. This target mimicry phenomenon with a coding RNA molecule is a new finding that has never been described before. *MtNSP1* promotion of *Mt*NSP2 expression would activate the mycorrhizal colonization because *Mt*NSP2 positively controls both the hyphal propagation along the root and the arbuscule formation. Finally, in tomato, *Sl-NSP1* itself would be directly or indirectly regulated by the AUX/IAA protein, *Sl*-IAA27. As a link with auxin this AUX/IAA protein is shown to be a new component of the signaling pathway controlling AM fungal colonization in tomato and is proposed to control strigolactone biosynthesis via the regulation of *Sl*-NSP1.

Overall our work has provided new pieces in the mycorrhizal puzzle and has shown how important it is to perform spatiotemporal investigations for a better understanding of highly integrated and complex biological processes.

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Summary:

The arbuscular mycorrhiza (AM), a symbiosis between fungi from the phylum *Glomeromycota* and nearly 80% of terrestrial plant species. It is characterized by a two-way exchange in which the fungus provides mineral nutrients to the plant in exchange for carbohydrates. However this "feeding" of the fungus during the symbiotic process represents a significant carbon cost for the plant. To maintain a mutualistic interaction the two symbiotic partners have to strictly control the extent of fungal development in the roots. This control is called autoregulation. Several proteins have been found to be important for the regulation of the different mycorrhizal steps: the stimulation of fungal growth in the rhizosphere by the strigolactones, the fungal entrance in the roots, the hyphal proliferation in the roots and the arbuscule formation.

In this work we examine in more detail the role of two of these proteins known to be involved in the mycorrhization process, the transcriptional factors NSP1 and NSP2 (Nodulation Signaling Pathway).

We first confirm in *M. truncatula* roots the direct implication of NSP1 in the regulation of two strigolactone biosynthesis genes, *DWARF27 (D27)* and *MAX1*, during the asymbiotic conditions. Then, we show that NSP1, unlike NSP2, is a factor that promotes the fungal entries in the root, presumably due to its activation of *D27* and *MAX1* resulting in a stimulation of strigolactone synthesis and presymbiotic fungal growth. Next, during the later stages of mycorrhization, we highlight that in the colonized tissues NSP1 is absent and the induction of both *D27* and *MAX1* is not anymore NSP1 dependent. NSP1 protein is then localized in cells which are not yet colonized but are close to a colonization zone. There, it controls negatively the hyphal propagation in the root and positively the formation of arbuscules. In contrast, NSP2 is present in the colonized tissue where it promotes hyphal propagation and arbuscule development, perhaps by interacting with other proteins.

We also show that if NSP1 proteins are absent of the colonized tissues, *NSP1* transcripts are present. Unexpectedly, we unveil that in those colonized cells, *NSP1* mRNA can protect, by a micro RNA (miR171h) decoy action called target mimicry, *NSP2* mRNA against miR171h-mediated degradation. This is the first demonstration that a coding RNA molecule can be a target mimic for a microRNA. In our context this finding reveals a positive regulation of NSP2 expression by *NSP1* transcripts and brings to light an additional layer of complexity in the mycorrhizal dual role of these two transcription factors.

Finally, in tomato, we highlight that *SlNSP1* could be directly or indirectly regulated by the AUX/IAA protein, *Sl*IAA27. As a link with auxin we presume that this AUX/IAA protein is a new component of the signaling pathway controlling AM fungal colonization in tomato, and we propose that it controls strigolactone biosynthesis via the regulation of *SlNSP1*.

Overall our work provides new pieces of the mycorrhizal puzzle and shows how important it is to perform spatiotemporal investigations for a better understanding of highly integrated and complex biological processes.

Key words: Arbuscular mycorrhizal symbiosis, Regulation, Strigolactones, NSP1, NSP2, D27, MAX1, miR171h, Medicago, Tomato, coding Target mimicry.

ED SEVAB : Interactions plantes-microorganismes.

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La symbiose mycorhizienne à arbuscule est une interaction bénéfique entre les champignons du phylum *Glomeromycota* et près de 80% des espèces de plantes terrestres. Elle est caractérisée par un échange réciproque de nutriments dans lequel le champignon fournit des sels minéraux à la plante en échange de sucres issus de la photosynthèse. Cependant, cette "alimentation" du champignon au cours de la symbiose représente un coût carbone important pour la plante. Ainsi, les plantes doivent strictement maitriser le développement des champignons symbiotiques dans les racines. Ce contrôle est appelé autorégulation. Plusieurs protéines ont été démontrées comme étant importantes pour la régulation des différentes étapes de la colonisation : la stimulation de la croissance fongique dans la rhizosphère par les strigolactones, l'entrée dans les racines, la prolifération des hyphes au sein des racines et la formation des arbuscules.

Dans ce travail, nous avons examiné plus en détail le rôle de deux de ces protéines connues pour être impliquées dans le processus de mycorhization, les facteurs de transcription NSP1 et NSP2 (Nodulation Signaling Pathway).

Nous avons d'abord pu confirmer dans les racines de *M. truncatula* en conditions non-symbiotiques, l'implication directe de NSP1 dans la régulation de deux gènes de biosynthèse des strigolactones, DWARF27 (*D27*) et MORE AXILLARY GROWTH (*MAX1)*. Ensuite, nous avons montré que NSP1, contrairement à NSP2, favorise l'entrée du champignon dans la racine, sans doute due à l'induction de la synthèse des strigolactones stimulant le champignon, via l'activation de *D27* et de *MAX1*. Ensuite, au cours des étapes ultérieures de la mycorhization, nous avons montré que dans les tissus colonisés, NSP1 est absent et que l'induction de *D27* et de *MAX1* n'était plus NSP1 dépendante. À cette étape, l'expression de la protéine NSP1 est localisée dans les cellules justes en amont du front de colonisation fongique. Là, elle contrôle négativement la propagation des hyphes dans la racine et positivement la formation des arbuscules. En revanche, NSP2 est présente dans le tissu colonisé où elle favorise la propagation des hyphes et le développement des arbuscules, peut-être en interaction avec d'autres facteurs.

Nous avons également montré chez *M. truncatula* que si les protéines NSP1 sont absentes des tissus colonisés, les transcrits de NSP1 sont présents. De façon inattendue, nous avons mis en évidence que l'ARN messager de NSP1 avait la capacité de protéger l'ARN messager de NSP2 contre sa dégradation par le microARN (miR171h), par une action de piégeage du miR171h, appelé effet mimicry. Ceci est la première démonstration qu'une molécule d'ARN codante peut être la cible mimétique d'un microARN. Dans notre contexte d'étude cette constatation révèle que les transcrits de NSP1 permettent une régulation positive de l'expression de NSP2, et met en lumière un niveau de complexité supplémentaire dans le rôle de ces deux facteurs de transcription dans la symbiose mycorhizienne.

Enfin, dans la tomate, nous avons montré que *Sl-NSP1* pourrait être directement ou indirectement régulée par une protéine AUX / IAA impliquée dans la réponse précoce à l'auxine, *Sl-IAA27*. Ce lien avec l'auxine nous fait présumer que cette AUX/AAI est un nouveau composant de la voie de signalisation du contrôle de la colonisation fongique dans la tomate, et nous proposons qu'il puisse avoir un rôle dans le contrôle de la biosynthèse des strigolactones via la régulation de *Sl-NSP1*.

L'ensemble de ce travail fournit de nouvelles pièces du puzzle constituant la symbiose mycorhizienne et montre l'importance de l'analyse des régulations spatiotemporelles pour une meilleure compréhension de ces processus biologiques extrêmement complexes.

Mots Clefs: Symbiose mycorhizienne, Arbuscule, Régulation, Strigolactones, NSP1, NSP2, D27, MAX1, IAA27, miR171h, Medicago, Tomate, coding Target mimicry.